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THE UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a dissertation entitled "Identification and Characterization of Proteins that Interact with the Human DNA Dependent Protein Kinase (DNA-PK)" submitted by Nicholas S.Y. Ting in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ABSTRACT

One of the key proteins required during cellular responses to DNA damage is the DNA dependent protein kinase (DNA-PK). DNA-PK is a serine/threonine protein kinase composed of a 465 kDa catalytic subunit called DNA-PKcs and a heterodimeric DNA-targeting component of 70 and 80 kDa, known as Ku. DNA-PK is activated by ends of double stranded DNA, *in vitro*, to phosphorylate substrates such as the tumor suppressor protein, p53, transcription factors, Sp1 and Serum Response Factor (SRF), the carboxy terminal domain (CTD) of RNA Polymerase II, and a protein required for DNA repair, XRCC4. Biochemical and genetic studies with various radiosensitive cell lines and mouse knockout models for Ku80, Ku70 and DNA-PKcs have shown that DNA-PK is required for the process of non-homologous end-joining during the repair of DNA double-strand breaks and V(D)J recombination.

During the initial purification of DNA-PK from Hela cells, several polypeptides that were phosphorylated in a DNA dependent manner were present in a partially purified active kinase fraction. Three of these polypeptides were identified as DNA-PKcs, Ku80 and Ku70. Similar polypeptides were observed to be present in a partially purified DNA-PK fraction from human placenta. Here, two of these polypeptides have been identified as the translation initiation factor, eIF-2 and a heterodimeric transcription factor, NF90/45. eIF-2β and NF90/45 are shown to be *in vitro* substrates of DNA-PK. Electromobility shift assays have demonstrated that a pool of polypeptides which contained eIF-2 and NF90/45 or recombinant NF90 alone was able to support the formation of a stabilized protein-DNA complex between purified DNA-PKcs and Ku on linear double stranded DNA. Moreover, antibodies to recombinant NF90 and NF45 immunoprecipitated DNA-PKcs, NF90 and NF45 from a partially purified protein fraction from human placenta and from crude cell extracts. However, Ku was not detected in these immune complexes. Based on these results, we propose that the interaction between NF90/45 and DNA-PKcs is required for the formation of a stable trimeric DNA-PKcs/Ku80/70 complex on linear double-stranded DNA, which may be important for the function of DNA-PK during non-homologous end-joining *in vivo*.

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List of Abbreviations

abbreviations

ADP	adenosine diphosphate
AFM	atomic force microscopy
ATP	adenosine triphosphate
ARRE	antigen receptor response element
ATM	mutated in Ataxia Telangiectasia
bp	base pairs
BSA	bovin serum albumin
BS^3	Bis(sulfosuccinimidyl) suberate
CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid
cDNA	complementary DNA
CT	calf thymus
CTD	carboxy terminal domain
DEAE	diethylaminoethyl
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	catalytic subunit of DNA dependent protein kinase
ds	double-stranded
dsb	double strand break
DTT	dithiothreitol
EDTA	ethylene-diamine-tetra-acetic acid
EGTA	ethylene glycol-bis(β-aminoethyl) ether
eIF-2	eukaryotic initiation factor-2
EM	electron microscopy
EMSA	electrophoretic mobility shift assay
FPLC	fast protein liquid chromatography
GDP	guanosine diphosphate
GTP	guanosine triphosphate

HAT	histone acetyltransferase
HCl	hydrochloric acid
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
Ig	immunoglobulin
IL-2	interleukin-2
KCl	potassium chloride
kDa	kilodalton
Ku70	70 kDa subunit of Ku protein
Ku80	80 kDa subunit of Ku protein (also referred to as Ku86)
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NF90	90 kDa nuclear factor
NF45	45 kDa nuclear factor
NHEJ	non homologous end-joining
PARP	poly (ADP-ribose) polymerase
PI-3	phosphatidylinositol-3
PMA	phorbol myristate acetate
PMSF	phenylmethylsulphonyl flouride
PVDF	polyvinylidene diflouride
RNA	ribonucleic acid
RPA	replication protein A
RT	room temperature
scid	severe combined immunodeficient
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH3	src homology domain 3
SPR	surface plasmon resonance
TBS	tris-buffered saline
TTBS	tris-buffered saline containig Tween20
TCR	T-cell receptor
V(D)J	variable (diversity) joining

amino acids

three letter code	amino acid name	single letter code
Ala	alanine	A
Arg	arginine	R
Asn	asparagine	N
Asp	aspartate	D
Cys	cysteine	C
Glu	glutamate	E
Gln	glutamine	Q
Gly	glycine	G
His	histidine	H
Ile	isoleucine	I
Leu	leucine	L
Lys	lysine	K
Met	methionine	M
Phe	phenylalanine	F
Pro	proline	P
Ser	serine	S
Thr	threonine	T
Trp	tryptophan	W
Tyr	tyrosine	Y
Val	valine	V

Greek letters

α	alpha
β	beta
γ	gamma
δ	delta
ε	epsilon

CHAPTER I

INTRODUCTION

Chapter I - Introduction

I.1: Protein phosphorylation

Eukaryotic cells respond to both external and internal stimuli in a rapid and highly regulated manner. These responses involve biochemical cascades that begin with specific sensors that sense and transmit the stimulus signal to transducers, which in turn stimulate specific targets to elicit the proper cellular response. Reversible covalent modification of proteins is one of the major means by which signals are transmitted in the cell. One of the predominant forms of covalent modification within the cell is the phosphorylation of proteins. The phosphorylation reactions catalyzed by protein kinases involve the transfer of a phosphate group from ATP onto a serine, threonine or tyrosine residue on proteins (Edelman *et al.*, 1987). Consequences of the phosphorylation event include alterations of the function and interaction specificity of the modified protein, two events that are crucial in transmitting signals. Therefore, protein kinases and phosphoproteins play crucial regulatory roles in a number of cellular response processes such as transcription, translation, mitogenic response pathways, and the control of the cell cycle (reviewed in Hunter, 1995a).

Protein kinases are activated by a number of factors depending on the specific cellular process in which they are involved. For example, receptor tyrosine kinases are activated by the presence of growth hormones during cellular proliferation, and the dsRNA dependent protein kinase (PKR) is stimulated by the presence of foreign viral RNA to regulate translation rates (Hunter, 1995a). The human DNA dependent protein kinase (DNA-PK), is activated *in vitro* by ends of double-stranded DNA (dsDNA) to phosphorylate proteins on serine or threonine residues (reviewed in Anderson and Lees-Miller, 1992; Lees-Miller, 1996). Ends of dsDNA can occur in the genome when, for example, cells become exposed to ionizing radiation or to certain chemical agents. Cells quickly respond by repairing the damaged DNA in order to maintain the integrity of the genome. Over the past five years, DNA-PK has been shown to play an important role in the maintenance of chromosome integrity within the eukaryotic cell (reviewed by Jeggo *et al.*, 1995; Lees-Miller, 1996; Jackson, 1997).

I.2: DNA dependent phosphorylation and the discovery of DNA-PK

In 1985, Anderson and coworkers reported the phosphorylation of several polypeptides when sonicated calf thymus DNA and $\gamma\ ^{32}P$-ATP were added to translation extracts from rabbit reticulocyte lysates (Walker *et al.*, 1985). Similar DNA induced protein phosphorylation was seen with cell extracts from various sources from human to clams. One of the proteins that was phosphorylated was identified as the 90 kDa heat shock protein, hsp90. Independently, Carter and colleagues also saw DNA dependent phosphorylation of casein using nuclear and whole cell Hela extracts (Carter *et al.*, 1988). Both laboratories proceeded to purify the novel kinase from Hela cells using hsp90 and casein as substrates, respectively (Lees-Miller *et al.*, 1990; Carter *et al.*, 1990). Protein kinase activity was found to be associated with a large polypeptide of approximately 350 kDa, as determined by its migration on denaturing polyacrylamide gels (SDS-PAGE). Lees-Miller and colleagues reported that the large polypeptide, initially called p350, could not be purified to homogeneity without the loss of kinase activity (Lees-Miller *et al.*, 1990). Instead, kinase activity was associated with a partially purified protein fraction that included p350 and several other polypeptides that were phosphorylated in a DNA dependent manner. One of these polypeptides was a heterodimer of 70 and 80 kDa subunits, which was identified as the human Ku autoantigen (Lees-Miller *et al.*, 1990). Ku is a very abundant nuclear protein and antibodies to Ku are found in patients with autoimmune diseases, Systemic Lupus Erthematosus and Scleroderma-Polymyosistis Overlap syndrome (Mimori *et al.*, 1981; 1986). Ku has a very high binding affinity for ends of dsDNA with a dissociation constant (K_d) of $1.5\text{-}4.0 \times 10^{-10}$ M (Blier *et al.*, 1993).

Early studies showed that homogeneous preparations of p350 or Ku did not have significant kinase activity. When approximately equimolar amounts of both monomeric p350 and dimeric Ku70/80 were combined with linear duplex DNA, protein kinase activity was stimulated 10 to 15 fold *in vitro* (Dvir *et al.*, 1993; Suwa *et al.*, 1994; Chan *et al.*, 1996). By DNAase footprinting and UV cross-linking experiments, Gottlieb and Jackson (1993) showed that p350 required Ku in order to be localized onto the same piece of DNA. Subsequent electrophoretic mobility shift assays (EMSA) and co-immunoprecipitation studies confirmed that Ku was required for targeting p350 to DNA (Dvir *et al.*, 1992; Suwa *et al.*, 1994; Chan and Lees-Miller, 1996). It was therefore

proposed that Ku binds to the ends of dsDNA and recruits p350 to form the active DNA-PK complex in a step-wise fashion. Recent findings from the Lieber and Chu research groups have put forth alternative mechanisms for DNA-PK activation (to be discussed later). Nevertheless, these initial studies showed that full DNA-PK activity requires p350, Ku, and linear duplex DNA (reviewed in Woodgett, 1993). Other DNA constructs were also found to be activators of DNA-PK. These included DNA purified from natural sources such as sonicated salmon sperm DNA, or chemically synthesized linear duplex oligonucleotides such as poly dI-dC with a minimal length of 16 bp (Carter *et al.*, 1990; Anderson and Lees-Miller, 1992). Closed-circular supercoiled plasmid DNA did not activate DNA-PK; in contrast, linearized plasmids with either blunt, 5' or 3' overhanging ends, are able to induce DNA-PK activity (Gottlieb and Jackson, 1993). Similarly, DNA constructs resembling dumbbells and hairpins with single-stranded extensions were capable of supporting Ku binding, and hence DNA-PK activity (Falzon *et al.*, 1993; Morozov *et al.*, 1994). DNA-PK activity was not supported by linear single-stranded DNA (ssDNA) or dsDNA with ends that were blocked by amino acids (Anderson and Lees-Miller, 1992).

I.3: Properties of p350 (DNA-PKcs)

Initial studies with purified p350 showed that it was capable of binding ATP analogues such as FSBA (flourosulfonyl benzoyladenosine), suggesting that p350 could be the catalytic subunit of DNA-PK (Anderson and Lees-Miller, 1992). In order to identify the gene encoding p350, purified p350 was initially digested with CNBr (cyanogen bromide) and the resulting peptides were purified to obtain amino acid sequence information. Degenerate oligonucleotide probes derived from the amino acid sequence were then used to screen a Hela cDNA library, and a 13 kb cDNA clone was later isolated and sequenced. The cDNA sequence predicted a 4096 amino acid polypeptide with a molecular mass of 465 kDa (Hartley *et al.*, 1995). The human p350 gene, called *PRKDC*, was localized to chromosome 8q11 and estimated to consist of about 100 exons spanning approximately 180 kbp (Jackson, 1997). To date, murine, equine and *Xenopus* genes for p350 have been identified (Danska *et al.*, 1996; Shin *et al.*, 1997; Labhart, 1997). Analysis of the amino acid sequence of human *PRKDC* showed a

region in the carboxy terminus of approximately 380 amino acid that contains a putative kinase domain that is related to the phosphatidylinositol-3 (PI-3) kinase superfamily (Figure I-1). Although this protein family is distinct from classical serine/threonine protein kinases, p350 and other PI-3 related kinase family members do contain the motifs DXXXXN and DFG. The amino acid residues in these motifs have been shown to play critical roles in catalysis for the classical serine/threonine protein kinases (reviewed in Hunter, 1995b). Mammalian members of the PI-3 related kinase family include human FRAP (for FKBP-Rapamycin associated protein), human TRRAP (for transformation/transcription domain-associated protein [McMahon *et al.*, 1998]) and ATM (for mutated in the human disease Ataxia Telangiectasia) (Figure I-1). Ataxia Telangiectasia (A-T) is a rare autosomal recessive genetic disorder, characterized by immune system deficiencies, growth retardation, neuronal degeneration, and a 100 fold increase in the incidence of some cancers such as leukemia and lymphoma (reviewed in Lavin and Shiloh, 1997). A number of *S. cerevisiae* gene products, Mec1p, Tel1p, Tor1p and Tor2p, also belong to the PI-3 related kinase family. Other members of this family include *S. pombe* RAD3, and *Drosophila* Mei-41 (Figure I-1) (Reviewed in Zakian, 1995; Abraham, 1996; Jackson, 1996).

The PI-3 kinase related family of protein kinases have two very conspicuous characteristics. First, their cDNA sequences predict polypeptides with molecular masses ranging from 300 kDa to 460 kDa. DNA-PKcs and TRRAP are two of the larger polypeptides within this family (Figure I-1; McMahon *et al.*, 1998). Second, although they are large polypeptides, their amino acid sequences bear little to no significant homology to any other known proteins except within the 400 amino acids of their C-termini, which contain sequences related to the PI-3 kinase protein. However, even within the putative PI-3 kinase domain, the amino acid similarity among the family members is weak. As illustrated in Figure I-1, the percentage of amino acids in this region that are identical to ATM ranges from 28% to 45%. Nevertheless, all members of the PI-3 kinase related protein kinase family have been shown to play crucial roles in cell cycle progression in response to genotoxic insults and mitogenic signals (reviewed in Hunter, 1995b; Keith and Schreiber, 1995; Abraham, 1996). This suggests that although the amino acid sequences may not be conserved among its members, functional

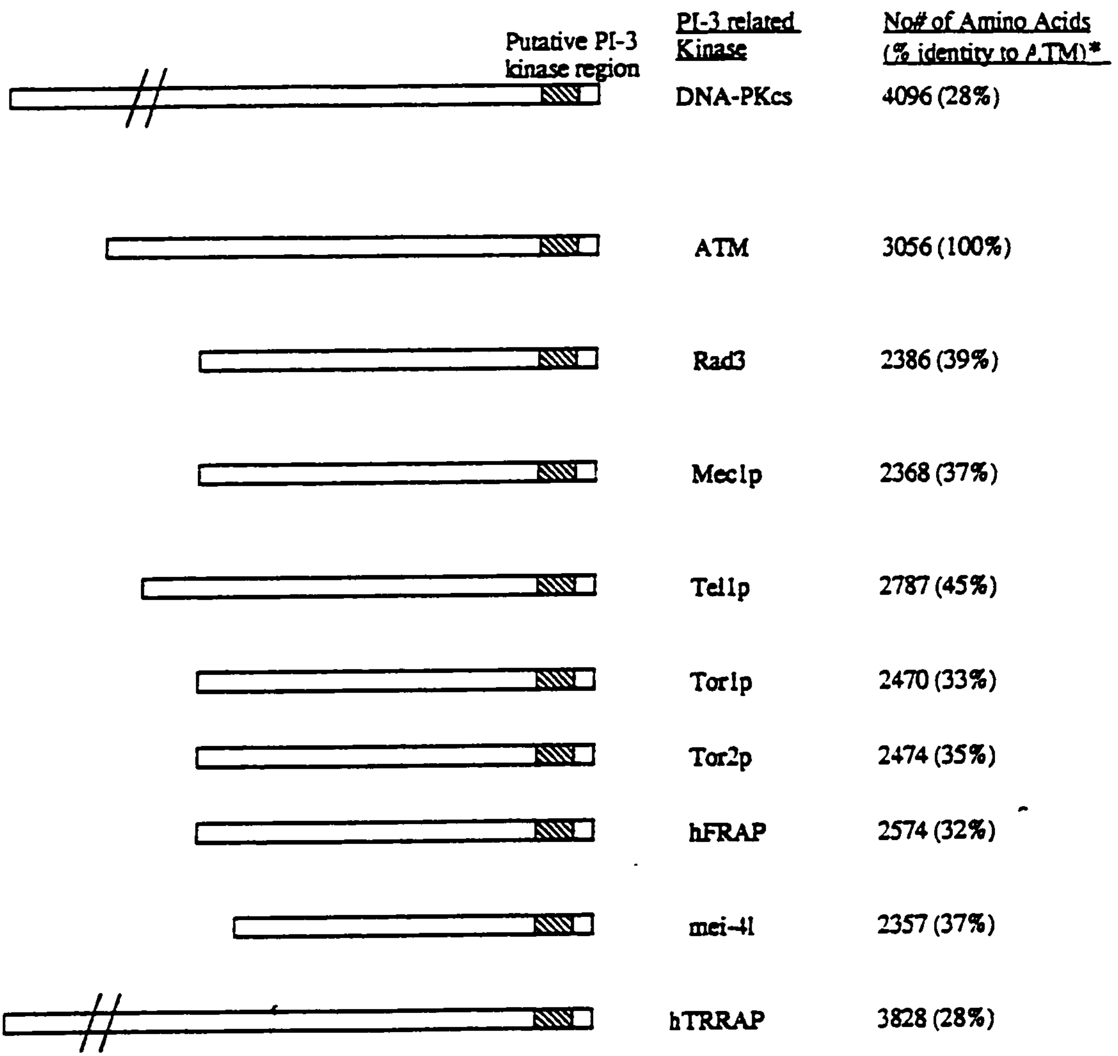

Figure I-1: The family of high molecular mass protein kinases containing the PI-3 kinase related catalytic domain (Adapted from Abraham, 1996; Hunter, 1995b, Jackson, 1996). The hatched box represent the putative PI-3 kinase domain. *The percent identity of the amino acid sequence in the putative PI-3 kinase domain of each gene product compared with the putative PI-3 kinase domain in the ATM protein is indicated in brackets. The number of amino acids for each gene product is also indicated. Mammalian gene products include DNA-PKcs, ATM, hFRAP, hTRRAP; *S. cerevisiae* gene products include Mec1p, Tel1p, Tor1p, Tor2p. Rad3 is a *S. pombe* gene product, while Mei-41 is a *Drosophila* gene product (reviewed in Abraham, 1996).

homology may be conserved within this family of PI-3 related kinases.

The identification of the human *PRKDC* gene confirmed the presence of a putative kinase domain in p350, and p350 has since been renamed DNA-PKcs for the catalytic subunit of DNA-PK. Although DNA-PKcs has amino acid sequence similarity to PI-3 kinases, no kinase activity towards phosphatidylinositol lipids has been detected to date. Recently, UV cross-linking, EMSA and atomic force microscopy (AFM) studies have shown that DNA-PKcs is capable of binding DNA in the absence of Ku (Yaneva *et al.*, 1997; Hammarsten and Chu, 1998). These same groups reported that highly purified DNA-PKcs possesses inherent kinase activity that can be further stimulated by Ku. These data further confirmed that DNA-PKcs is the catalytic subunit of DNA-PK. Relevance of these observations, in regards to the mechanism of activation of DNA-PK, will be discussed later.

I.4: Properties of Ku

Ku is a nuclear DNA-binding protein comprised of 70 and 80 kDa subunits that exist at approximately 5 x 10^5 molecules per cell (reviewed in Dynan and Yoo, 1998). The human genes for both Ku70 and Ku80 have been cloned and have been mapped to chromosome 22q13 and chromosome 2q33-q34, respectively (Cai *et al.*, 1994). Ku homologues have been reported in yeast, *Xenopus*, *Drosophila*, *Caenorhabditis* and rodents (reviewed in Dynan and Yoo, 1998). The subunits of the Ku protein are tightly associated and do not separate during protein purification. Using yeast two hybrid assays, the interface of heterodimerization between the two subunits has been localized to a 20 kDa region in the C-terminus of Ku70 and a 32 kDa region in the C-terminus of Ku80 (Wu and Lieber, 1996; Osipovich *et al.*, 1997). Ku70 alone has been shown to bind DNA by South-western blotting; however, in EMSA studies with crude cell extracts or purified proteins, both subunits are required for DNA end-binding activity (Rathmell and Chu, 1994a,b; Nussenzweig *et al.*, 1996; Bliss and Lane, 1997). This suggests that DNA binding of Ku *in vivo* requires both subunits.

Ku has a high binding affinity for ends of duplex DNA, with no preference for blunt, 5' or 3' overhanging ends. The DNA binding characteristics of Ku have been extensively studied by EMSA (Mimori and Hardin, 1986; de Vries *et al.*, 1989; Paillard

and Strauss, 1991; Blier *et al.*, 1993). Based on these studies, it has been proposed that Ku binds to the ends of the DNA and translocates to internal sites on the DNA in an ATP-independent fashion (de Vries *et al.*, 1989). This allows binding of multiple Ku molecules to a single DNA fragment. Depending on the amount of Ku and the length of the DNA fragment, these multimeric Ku-DNA complexes can be visualized as a ladder of bands in EMSA (Blier *et al.*, 1993), or as "beads on a string" by electron microscopy (de Vries *et al.*, 1989; Cary *et al.*, 1997). Recent AFM studies demonstrated that under certain conditions, DNA bound Ku promoted further Ku protein interactions, leading to the formation of DNA loop structures (Cary *et al.*, 1997). Other DNA structures that supported Ku binding included duplex DNA ending in hairpin loops and DNA constructs in which an internal segment of duplex DNA is flanked by ssDNA extensions (Falzon *et al.*, 1993). In EMSA studies, circular mini-duplex molecules containing a 30-nucleotide single-stranded region (gap) or a double-stranded segment of non-homologous sequence (bubble) competed well with linear duplex DNA for Ku binding (Falzon *et al.*, 1993). Ku was also capable of binding to linear ssDNA, but not with the same avidity as to linear dsDNA.

Many groups have isolated and characterized proteins that appear to be similar, if not identical to Ku. These Ku analogues or Ku have been isolated as putative transcription factors that interact with specific DNA sequences. These Ku analogues include PSE-1 which contacts the U1 snRNA promoter (Knuth *et al.*, 1990), TREF which binds specifically to the human transferrin gene promoter (Roberts *et al.*, 1994), and E_1BF which acts as a positive regulator for RNA polymerase I transcription initiation (Hoff *et al.*, 1994). More recently, Hache and colleagues reported that Ku binds to the negative regulatory element sequence (NRE1) in the long terminal repeat of the mouse mammary tumor virus (MMTV) and recruits DNA-PKcs activity to repress viral transcription (Giffin *et al.*, 1996). These findings initiated a debate of whether or not Ku could be involved in the control of transcription of certain genes by binding to specific DNA sequences.

This issue was addressed elegantly by the work of Bliss and Lane (1997). By using competitive EMSA studies, they showed that when bound to one DNA molecule, Ku could transfer directly to another DNA molecule if the two DNA molecules had

complementary overhanging ends containing a minimum of four matched bases. This observation can give a false impression of Ku binding to a specific DNA sequence under certain assay conditions. For instance, in EMSA competition assays when DNA probe (radiolabeled) with a specific internal sequence of interest was added to Ku, and then competed with DNA (unlabeled) having a mutated internal sequence of interest but with non-matching overhanging ends, this resulted in no competition of Ku binding. However, if unlabeled competitor DNA that was identical to the labeled DNA probe was used, addition of the unlabeled competitor DNA to the pre-incubated reaction containing Ku and DNA probe, did result in competition for Ku binding. Thus, competition of Ku binding in this situation was not dictated by the specificity of the internal DNA sequence, but by the presence of complementary overhanging ends. The order of addition was important as well; when both competitor and probe DNA were incubated together before the addition of Ku, regardless of the internal and overhanging sequences, competition of Ku binding was seen. Based on these results, Bliss and Lane (1997) proposed two conditions that must be met to show sequence specific binding of Ku using competitive EMSA. First, the competitor DNA (unlabeled) and probe DNA (labeled) must be identical in sequence (except for any mutated sequences to be tested) and must be added to the reaction before Ku. Second, it must be established that Ku can recognize the proposed specific sequence in the context of a closed-circular plasmid. Bliss and Lane (1997) also cautioned that highly homogeneous preparations of Ku must be used in these studies to eliminate the possibility that sequence specificity may be conferred through Ku-interacting proteins. By these criteria, the most convincing evidence for sequence specific binding of Ku came from the work of Hache and colleagues (Giffin *et al.*, 1996). They demonstrated that a plasmid harboring the NRE1 site from the MMTV was not only able to support Ku binding, but also activated DNA-PK activity, whereas a control plasmid lacking this site had no effect. When linearized, both plasmids were able to activate DNA-PK.

Ku has also been reported to have ATP dependent DNA helicase activity (Tuteja, *et al*, 1994), while others report Ku does not have helicase activity, but possesses DNA dependent ATPase activity (Cao *et al*, 1994). Relevance of these data in regards to the functions of DNA-PK will be discussed later.

I.5: Properties of DNA-PK

a) What stimulates DNA-PK activity?

In vitro, full DNA-PK activity requires approximately equimolar amounts of DNA-PKcs and Ku, and ends of an appropriate dsDNA construct (reviewed in Lees-Miller, 1996; Jackson, 1997; Dynan and Yoo, 1998). Double-stranded DNA constructs that have been shown to stimulate DNA-PK activity to date are illustrated in Figure I-2 (reviewed in Anderson, 1994; Lees-Miller, 1996; Dynan and Yoo, 1998). Although Ku can bind ssDNA, it does not activate DNA-PK. In fact, addition of ssDNA to reactions containing dsDNA, inhibits DNA-PK activity (Lees-Miller *et al.*, 1990; Anderson and Lees-Miller, 1992).

In the literature, there is debate whether or not DNA-PK can be activated by plasmids with a single-stranded gap or with a single nick. Initial studies showed that extensively purified plasmids (>3000bp) nicked with DNAase I or restriction enzymes in the presence of ethidium bromide (EtBr) did not induce DNA-PK activity (Gottlieb and Jackson, 1993). On the other hand, Kuff and colleagues reported that synthetic mini-circles of 350 bp with a single nick, or ssDNA gaps in which 6 or 30 nucleotides are missing, did activate DNA-PK (Morozov *et al.*, 1994) (Figure I-2). It has been proposed that these differing results may be due to the presence of residual intercalated EtBr that could interfere with protein-DNA binding (Anderson, 1994). The results of Gottlieb and Jackson (1993) were recently corroborated in two separate reports (Weinfeld *et al.*, 1997; Smider *et al.*, 1998). Furthermore, Weinfeld and colleagues reported that plasmid DNA (pUC18 plasmid, >3000bp) irradiated with X-rays at doses that produced single nicks, did not activate DNA-PK as well as plasmids irradiated with doses that created double-strand breaks (Weinfeld *et al.*, 1997). Smider and colleagues also showed that a 68 bp DNA construct with two hairpin ends that contains a single nick did not activate DNA-PK (Smider *et al.*, 1998). It was contended that the results of Kuff and colleagues (Morozov *et al.*, 1994) could be attributed to peculiar features of the 350 bp mini-circle (Smider *et al.*, 1998); for DNA fragments less than 500 bp, the intrinsic rigidity of DNA may inhibit its covalent closure resulting in possible structural abnormalities at the site of the nick (Smider *et al.*, 1998). Nevertheless in all cases, DNA-PKcs, Ku and ends of an appropriate dsDNA construct were required for kinase activity. Combining these

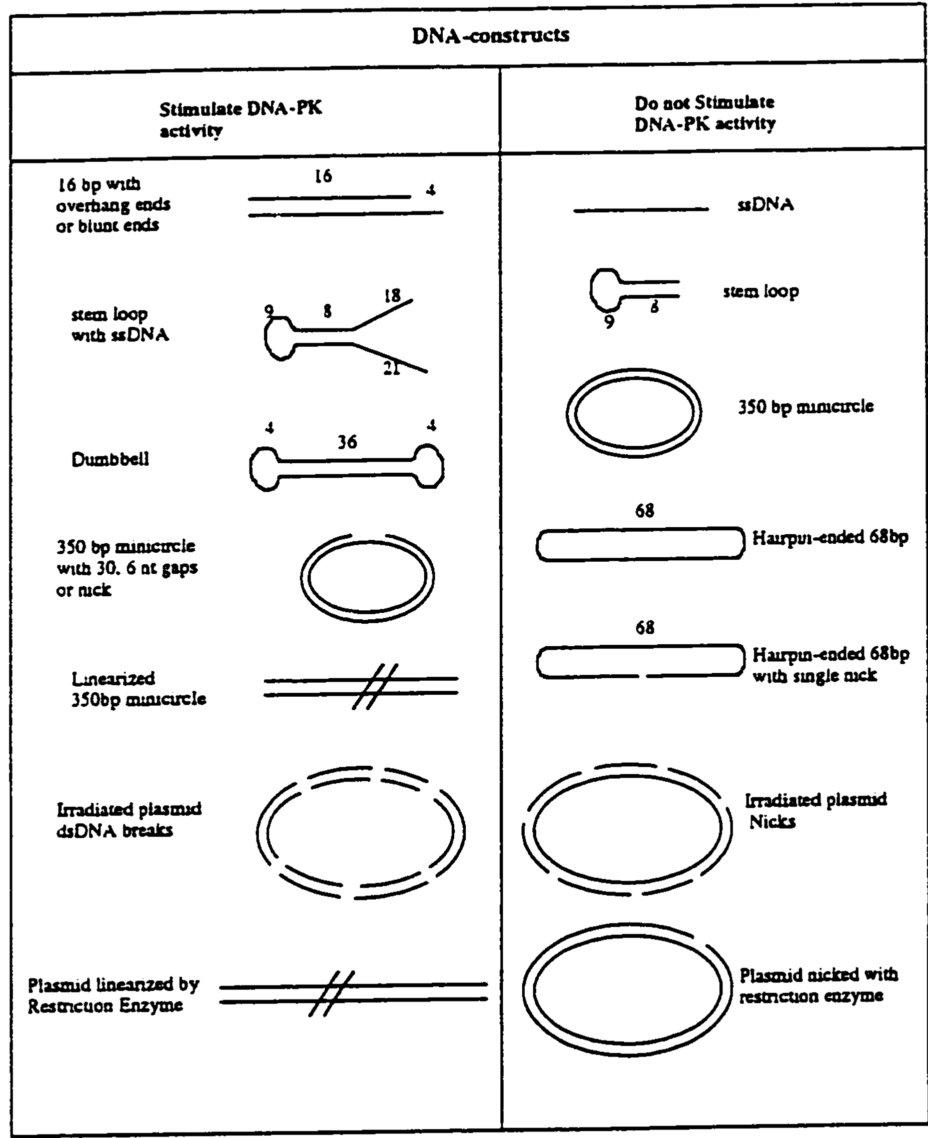

Figure I-2: DNA constructs that stimulates DNA-PK activity *in vitro* (adapted from Morozov *et al.*, 1994; Anderson, 1994; Weinfeld *et al.*, 1997). Ku binds to linear dsDNA with blunt, 5' or 3' overhanging ends. A minimum of length of 16 bp linear dsDNA is required for DNA-PK activity. Stem loop with ssDNA extensions and dumbbell DNA structures all support Ku binding and DNA-PK activity. According to Kuff and colleagues, 350 bp mini-circle with single stranded gaps of 30, 6 or a single nick are activators of DNA-PK. On the other hand, plasmid DNA nicked with restriction enzymes in the presence of EtBr do not activate DNA-PK (Gottlieb and Jackson, 1993; Weinfeld *et al.*, 1997). Furthermore, plasmids irradiated with an X-ray dose that only result in nicks do not activate DNA-PK as well as plasmid irradiated with an X-ray dose that causes ds breaks (Weinfeld *et al.*, 1997). Other constructs that do not activate DNA-PK include stem loop structure, ssDNA and intact mini-circles (350 bp) or closed circular plasmids. Moreover, a 68 bp DNA construct with hairpin ends with or without a single nick do not activate DNA-PK (Smider *et al.*, 1998).

studies with the early immunoprecipitation, UV cross-linking, EMSA and DNAase footprinting data, suggest the following model of how DNA-PK becomes activated *in vitro*: Ku initially binds to the ends of duplex DNA and recruits DNA-PKcs to form the active DNA-PK complex (reviewed in Lees-Miller, 1996; Jackson, 1997; Dynan and Yoo, 1998).

Recent reports that showed DNA-PKcs binds DNA and has kinase activity in the absence of Ku *in vitro*, have challenged the proposed model for DNA-PK activation. Utilizing UV cross-linking, EMSA, and atomic force microscopy (AFM), Lieber and coworkers concluded that highly purified DNA-PKcs is capable of binding 18 bp linear dsDNA and this association was enough to stimulate kinase activity (Yaneva *et al.*, 1997). DNA-PKcs activity was further stimulated by Ku only with linear dsDNA longer than 30 bp. Under these conditions, AFM imaging indicated that DNA-PKcs binds at the DNA termini, and Ku assumes a position along the dsDNA that is adjacent to DNA-PKcs. Hammarsten and Chu (1998) reported similar Ku independent activation of DNA-PKcs kinase and DNA binding activity with a linear ds 32 bp synthetic oligonucleotide. However, two caveats exist regarding these studies. First, the ATP concentration utilized in the DNA-PK activity assays used by Lieber and colleagues was only 10 nM (Yaneva *et al.*, 1997). This is 2500 times less than the reported Km of DNA-PK for ATP which is 25 μM (Anderson and Lees-Miller, 1992). Second, the salt concentration in the EMSA binding assays employed by Hammarsten and Chu (1998) was 10 mM KCl, which is 14 times lower than the estimated salt concentration that exists within the eukaryotic cell (140 mM, includes K^+ and Na^+ ions [Alberts *et al.*, 1989]). Although the assay conditions employed in these studies may not be physiologically relevant, these data certainly suggest alternative mechanisms for DNA-PK activation *in vitro*. One possibility is that the only function of Ku is to make the DNA more accessible for the binding and activation of DNA-PKcs, irrespective of a direct contact between the two proteins (Yaneva *et al.*, 1997) The reported helicase activity of Ku may play a role in this regard (Tujeta *et al.*, 1994).

Other studies indicate that DNA-PK kinase activity can be stimulated by other proteins. A novel protein called C1D, stimulates DNA-PK kinase activity in the absence of dsDNA termini when C1D is associated with closed-circular supercoiled plasmid

DNA (Yavuzer *et al.*, 1998). As well, the heat shock transcription factor 1 (HSF1) (Huang *et al.*, 1997) and the chromatin associated HMG (high mobility group) proteins 1 and 2 have also been shown to stimulate DNA-PK activity *in vitro* (Watanabe *et al.*, 1994). In each of these cases, Ku is still required for DNA-PK activity.

Ku independent stimulation of DNA-PKcs activity has been shown with the DNA damage sensor protein, poly ADP-ribose polymerase (PARP) (Ruscetti *et al.*, 1998). PARP utilizes NADP to catalyze the production of ADP-ribose polymers in the presence of DNA damage. In the presence of NADP, PARP enhances the phosphorylation of a substrate hnRNP-U (heterogeneous nuclear ribonucleoprotein-U) by purified DNA-PKcs *in vitro* (Ruscetti *et al.*, 1998). This suggests that modification of DNA-PKcs by PARP stimulates DNA-PKcs activity. PARP also stimulates DNA-PK activity (DNA-PKcs and Ku) towards p53 and the replication protein A (RPA) *in vitro* (Ruscetti *et al.*, 1998). The relevance of these findings will be discussed in context of DNA-PK's role in DNA repair.

b) What inhibits DNA-PK activity?

Besides ssDNA, DNA-PK activity is inhibited by pyrophosphate (Anderson and Lees-Miller, 1992), 6-dimethyl-aminopurine (DMAP) (Kuhn *et al.*, 1995; Labhart, 1995), a pyridone derivative, OK-1035 (Take *et al.*, 1995) and the sterol-like fungal metabolite, wortmannin (Hartley *et al.*, 1995). Wortmannin inhibits 50% of PI-3 kinase activity (IC50) towards PI-3 lipid substrates at a concentration of 20 nM. The IC50 of wortmannin that is required to inhibit DNA-PK is 10 times higher at 200 nM (Hartley *et al.*, 1995). In spite of this, wortmannin remains to be one of the more effective inhibitors of the PI-3 related kinase family. For example, ATM kinase activity has recently been shown to be also inhibited by wortmannin at an IC50 concentration of 100 nM (Banin *et al.*, 1998). DNA-PK is also inhibited by autophosphorylation (Chan and Lees-Miller, 1996). In the absence of a suitable substrate, all three protein components become phosphorylated. Under these conditions, phosphorylated DNA-PKcs and phosphorylated Ku can no longer be co-immunoprecipitated in the presence of DNA, suggesting that phosphorylation of DNA-PKcs results in its dissociation from Ku bound DNA, leading to the inactivation of the kinase (Chan and Lees-Miller, 1996). Finding ways to inhibit DNA-PK activity remain a crucial strategy for studying DNA-PK function *in vivo*.

I.6: Substrates of DNA-PK

Over the past 5 years, a number of *in vitro* substrates have been identified for DNA-PK (Anderson and Lees-Miller, 1992; Anderson, 1993; Lees-Miller, 1996). The DNA-PK substrates listed in Table I-1 are classified as either DNA binding or non-DNA binding proteins. The DNA-PK phosphorylation sites on the substrates (if identified) are also listed in Table I-2. Casein and hsp90 were the substrates initially used to purify DNA-PK from Hela cells (Lees-Miller *et al.*, 1990; Carter *et al.*, 1980). Along with hsp90, DNA-PK phosphorylation sites on SV40 large T antigen, serum response factor (SRF), the tumor suppressor protein p53 and c-jun were mapped to determine if a consensus phosphorylation site exists. It was evident that in each of these substrates, DNA-PK preferentially phosphorylated serine or threonine that was followed by a glutamine (SQ or TQ). Based on these results, a peptide (called the SQE peptide) containing the S/TQ motif was developed to assay for DNA-PK activity (Lees-Miller *et al.*, 1992). The peptide EPPLSQEAFADLWKK, derived from the DNA-PK phosphorylation site on the amino-terminus of the p53, proved to be an excellent substrate for DNA-PK. Altering the glutamine (Q) residue following the serine or threonine to a glutamate (E) yielded a peptide that was not a DNA-PK substrate (Lees-Miller *et al.*, 1992). Recently, a derivative of this peptide, PESQEAFADLWKK, was shown to be at least twice as efficient when compared to the original peptide under the same assay conditions (Anderson *et al.*, 1995). The SQE peptide remains as the hallmark peptide used to assay for DNA-PK activity *in vitro*.

As the number of DNA-PK phosphorylation sites that were mapped increased, it became apparent that "non-S/TQ" sites also exist (Table I-1). For example, DNA-PK phosphorylates c-fos on Ser288 in the sequence RSVP, and the carboxy terminal domain (CTD) of RNA polymerase II at Ser7 of the heptad repeat, YSPTSPS (Anderson and Lees-Miller, 1992). In addition, data from our laboratory showed that DNA-PK phosphorylation sites on Ku80 and Ku70 occur on sites that are not followed by glutamine (Chan *et al.*, 1999; in press). For many other substrates, including DNA-PKcs itself, the site of phosphorylation has not been identified. Although all of the DNA-PK substrates contain S/TQ motifs, it is very likely that DNA-PK will target other sites.

Table I-1: DNA-PK substrates identified to date (Adapted from Lees-Miller, 1996; Anderson, 1993). Substrates are divided into DNA binding and non-DNA binding proteins. The majority of the DNA-binding substrates are known transcription factors, while the non-DNA binding substrates are proteins that are associated with various cellular functions. The DNA-PK phosphorylation sites on each substrate are indicated if they are identified.

DNA-PK substrates	**Identified phosphorylation site.**	**Reference**
	DNA binding proteins	
SV40 large tumor antigen (T-Ag)	Ser677	Chen *et al.*, 1991
Tumor suppressor p53	Ser15 and Ser37	Lees-Miller *et al.*, 1992; Ficsella *et al.*, 1993
Ku (70 and 80 kDa)	Ser5; Ser577, 580 and Thr715	Chan *et al.*, 1998; in press
DNA-PKcs		Lees-Miller *et al.*, 1990; Carter *et al.*, 1990
RNA polymerase II CTD domain	Ser7 of Heptad Repeat	Anderson and Lees-Miller, 1992
Serum Response Factor (SRF)	Ser435, Ser446	Liu *et al.*, 1993
cJun	Ser249	Bannister *et al.*, 1993
cFos		Abate *et al.*, 1993
Oct-1 and -2		Anderson *et al.*, 1995; Giffin *et al.*, 1996
Sp1		Gottlieb and Jackson, 1993
cMyc		Iijima *et al.*, 1992
TFIIB		Chibazakura *et al.*, 1997
TATA binding protein, TBP		Labhart, 1996
Progesterone Receptor		Weigel *et al.*, 1992
Replication Protein A (34 kDa)		Brush *et al.*, 1994; Pan *et al.*, 1994; Boubnov *et al.*, 1995
Histone H2A.X		Kleinschmidt and Steinbeisser, 1991
Xenopus P1 protein		Someya *et al.*, 1995
Poly ADP-ribose polymerase (PARP)		Ruscetti *et al.*, 1998
Glucocorticoid receptor	Ser527	Giffin *et al.* 1996, 1997
	Non-DNA binding proteins	
Microtubule associated protein Tau		Wu *et al.*, 1993
Casein		Carter *et al.*, 1988
90 kDa heat shock protein (hsp90α)	Thr2, Thr4	Lees-Miller and Anderson, 1989
XRCC4		Leber *et al.*, 1998
IκBα	Ser36, Thr273	Liu *et al.*, 1998
human GCN5		Barlev *et al.* , 1998
hnRNP-U		Ruscetti *et al.*, 1998

With certain DNA binding substrates, such as Sp1 and p53, it has been observed that increasing the concentration of DNA inhibits substrate phosphorylation by DNA-PK. Phosphorylation of hsp90 is unaffected when incubated with Sp1 or p53 and DNA-PK at increasing concentrations of DNA. This suggests that for DNA-binding substrates such as p53 and Sp1, co-localization of both substrate and DNA-PK on the same piece of DNA fragment is required for efficient phosphorylation of the substrate (Jackson *et al.*, 1990; Lees-Miller *et al.*, 1992; Anderson and Lees-Miller, 1992).

Despite the large number of DNA-PK substrates that have been identified *in vitro*, the definitive identification of physiologically relevant DNA-PK substrates has been elusive. Data in the literature suggest that Ku and p53 are likely *in vivo* substrates of DNA-PK. Ku has been purified from ^{32}P-orthophosphate *in vivo*-labeled cells by immuno-affinity chromatography and shown to be a phosphoprotein that is phosphorylated at serine residues (Yaneva *et al.*, 1985). Interestingly, phosphorylation of Ku was shown to be reduced in extracts from *scid* cells (severe combined immunodeficient) (Boubnov and Weaver, 1995), which have very low levels of DNA-PK activity (to be discussed later). *In vitro*, autophosphorylation of Ku also occurs on serine and threonine residues (Chan *et al.*, 1999; in press); whether or not these are the same sites *in vivo* remains to be determined.

The tumor suppressor protein p53, was one of the first DNA-PK substrates to be identified. DNA-PK phosphorylation sites have been mapped to Ser15 and Ser37 in the amino-terminus of p53 (Lees-Miller *et al.*, 1992). Transformed cells that were transfected with vectors expressing wild type p53 or p53 in which Ser37 had been changed to Ala, arrested at S-phase of the cell cycle. Conversely, cells expressing p53 in which Ser15 had been changed to Ala did not block cell cycle progression as well as wild type p53. These results indicated that p53 was a potential *in vivo* target for DNA-PK, and that phosphorylation of Ser15 may be important for p53's ability to inhibit cell cycle progression (Fiscella *et al.*, 1993).

One of the most important goals in the field of protein kinase research is to address whether or not the phosphorylation event alters the function of the substrate. This problem is extremely difficult to answer if the function of the substrate has not been unequivocally defined. Over the past three years, our understanding of the function of

DNA-PK substrates such as p53 has grown tremendously. At the same time our knowledge of the role of DNA-PK in the eukaryotic cell has increased. It is therefore appropriate to discuss the consequences of DNA-PK phosphorylation in the context of the proposed physiological role of DNA-PK and the substrate.

I.7: Function of DNA-PK

The biochemical properties of DNA-PK and the substrates identified for DNA-PK, suggested that DNA-PK may be involved in the detection or repair of damaged DNA and in DNA recombination. These speculations were confirmed with one crucial observation made independently by Getts and Stamato (1994) and Rathmell and Chu (1994a) with the *xrs* (X-ray sensitive) hamster cells. Follow-up reports from the research laboratories of Jeggo, Jackson and Alt presented genetic and biochemical evidence to unequivocally show that DNA-PK is required for DNA double-strand break (dsb) repair and V(D)J recombination (reviewed by Jeggo *et al.*, 1995; Jackson and Jeggo, 1995; Jackson, 1996).

a) DNA repair in eukaryotic cells

The eukaryotic genome is constantly exposed to intrinsic and extrinsic factors that can lead to DNA damage. There are four general types of DNA damage that are repaired by four specific DNA repair pathways (reviewed in Friedberg *et al.*, 1995). First, deamination of bases, for example of cytosine to uracil, can occur spontaneously and is repaired by the base excision repair pathway. Second, exposure to ultraviolet rays (UV) can cause formation of cyclobutane pyrimidine dimers which are repaired by the nucleotide excision repair pathway (NER). Third, mismatched bases incorporated during DNA replication are corrected by the mismatch repair system. Lastly, oxygen radicals generated from exposure to ionizing radiation (X-rays and γ-rays) can lead to double strand DNA breaks which are repaired by the DNA double strand break repair machinery (reviewed in Friedberg *et al.*, 1995).

The potentially most lethal form of these general types of DNA damage are DNA double-strand breaks (dsbs). Besides exposure to irradiation in the form of X-rays or γ-rays, DNA dsbs can be caused by drugs that generate oxygen free radicals such as

bleomycin, and drugs that inhibit topoisomerase II, such as etoposide and adriamycin, which create dsbs in the form of protein-DNA intermediates. DNA dsbs can also occur during normal cellular activities such as V(D)J recombination, DNA replication and mitosis (reviewed in Weaver, 1996; Chu, 1997). Failure to repair DNA dsbs can lead to cell death and improper repair of DNA dsbs can lead to chromosome translocations and loss of genetic information which can easily lead to tumorigenesis.

DNA dsbs in eukaryotic cells are repaired by either homologous recombination or non-homologous end-joining (NHEJ) (reviewed by Friedberg *et al.*, 1995; Weaver, 1996; Chu, 1997). DNA dsb repair via homologous recombination relies on the information provided by a second chromosome copy that is used as a template to repair the damaged region. This mechanism is favored in lower eukaryotes such as the budding yeast. DNA dsb repair by NHEJ involves the ligation of two DNA double strand molecules with exposed ends without the use of an adjacent homologous sequence of chromosomal DNA (reviewed by Weaver, 1996; Chu, 1997; Lieber *et al.*, 1997).

Early studies within the field of DNA dsb repair relied on hamster or mouse cell lines that are sensitive to ionizing radiation in the form of X-rays or γ-rays. These mutant cells, termed *xrs* or *irs* for X-ray or irradiation sensitive, had a range of phenotypes, with some displaying only minor radiosensitivity to X-rays, while others displayed a dramatic response to other DNA damaging agents. Efforts to identify the genetic defect(s) in these cells resulted in the establishment of eleven genetic complementation groups (reviewed in Zdzienicka, 1995; 1996). Interestingly, cells belonging to four complementation groups (4, 5, 6 and 7) were severely compromised in their ability to repair genomic DNA dsbs as indicated by their extreme sensitivity to ionizing radiation, with little cross-sensitivity to UV or alkylating agents. Human genes identified by their ability to restore X-ray resistance within these four groups were called X-ray cross-complementing or *XRCC4, 5, 6* and *7* (reviewed in Jeggo *et al.*, 1995).

b) A connection between V(D)J recombination and DNA dsb repair

The process of V(D)J recombination also generates DNA double-strand breaks. V(D)J recombination involves rearranging specific DNA sequences to create a genetically diverse repertoire of immunoglobulin (Ig) and T-cell receptor (TCR) genes

during the development of T and B lymphocytes in mammals. This genetic repertoire is required to combat the multitude of antigens encountered by the immune system. The first genes identified to be required for V(D)J recombination were the lymphoid specific recombination activating genes RAG1 and RAG2 (reviewed in Lewis, 1994). The possibility that V(D)J recombination also required the DNA dsb repair pathway was first realized when lymphoid and non-lymphoid cells from severe combined immunodeficient (*scid*) mice were shown to be sensitive to ionizing radiation (Fulop and Phillips, 1990; Hendrickson, *et al.*, 1991). *Scid* mice lack mature T and B lymphocytes due to their inability to carry out V(D)J recombination. Genetic complementation studies placed *scid* cells into X-ray complementing group 7 (*XRCC7*). Cell lines belonging to XRCC groups 4, 5, and 6 were then tested for their ability to carry out V(D)J recombination. These tests, which are called transient recombination assays, involved transfecting cells with the recombination activating genes, RAG1 and RAG2, and an appropriate rejoining DNA substrate. None of the cells in the four groups were successful in producing the appropriate product (indicative of a wild type V(D)J recombination event), suggesting that these cells are deficient for both V(D)J recombination and DNA dsb repair (reviewed in Jeggo and Jackson, 1995; Jeggo *et al.*, 1995; Jackson, 1996).

I.8: DNA-PK, DNA dsb repair and V(D)J recombination.

a) Lessons from the XR complementation groups.

In 1994, the research groups of Stamato and Chu independently reported that cell extracts derived from *xrs6* (XRCC group 5) hamster cell lines lacked DNA duplex end-binding activity (Getts and Stamato, 1994; Rathmell and Chu, 1994a). The end-binding activity present in the parental wild type (CHO-K) cell extracts was super-shifted by antibodies to Ku70 (Getts and Stamato, 1994), suggesting that the Ku80/70 heterodimer was responsible for the DNA end-binding activity seen in crude cell extracts. This suspicion was confirmed when the human gene *XRCC5* was localized to chromosome locus 2q33-35, which included the Ku80 gene. The Ku80 cDNA was then shown to complement the radiosensitivity and V(D)J recombination defects of the *xrs6* cell line, confirming that Ku80 is the product of the *XRCC5* gene (Taccioli *et al.*, 1994; Rathmell and Chu 1994b; Smider *et al.*, 1994; Boubnov *et al.*, 1995).

Logically, these results triggered the question of whether or not any of the other three *XRCC* genes (*XRCC4, 6* and *7*) encoded other components of DNA-PK (Table I-2). *Sxi-1* hamster cells, which belong to XRCC group 6, lack DNA end-binding activity, raising the possibility that this cell line could be defective for Ku70 (Peterson *et al.*, 1995b). However, the precise genetic defect(s) in these cells has yet to be determined. Recently, murine embryonic stem (ES) cells, carrying an inactivated *Ku70* gene, have been shown to be extremely radiosensitive, to lack DNA end-binding activity and to be unable to carry out V(D)J recombination in transient recombination assays (Gu *et al.*, 1997).

Interestingly, cells from the XRCC4 and XRCC7 complementing groups had normal levels of Ku-dependent end-binding activity (Li *et al.*, 1995; Peterson *et al.*, 1995b). The *XRCC4* gene has been cloned and its cDNA predicts a novel protein of 37 kDa with no amino acid sequence homology to any other known protein (Li *et al.*, 1995). Its function has not been precisely defined, but transfection of its cDNA into the mutant hamster cell line *XR-1* restored X-ray resistance, and co-transfection with RAG1 and RAG2 cDNA reinstated normal V(D)J recombination activity (Li *et al.*, 1995). Recent studies have shown that XRCC4 interacts with DNA-PK and with DNA Ligase IV, suggesting that XRCC4 may act as a bridging molecule that tethers DNA-PK and DNA Ligase at a DNA damage site (Grawunder *et al.*, 1997; Leber *et al.*, 1998) (to be discussed later).

Finally, three lines of evidence suggest that the *XRCC7* gene encodes DNA-PKcs. First, cell extracts from the two mutant cell lines assigned to this group, the *V3* hamster and *scid* mouse cells, fail to show DNA-PK activity and do not have a protein that cross-react with antibodies to DNA-PKcs (Blunt *et al.*, 1995; Kirchgessner *et al.*, 1995; Peterson *et al.*, 1995b). Second, the candidate genes for DNA-PKcs, *XRCC7*, and the gene complementing *scid* cells, map to the same region of human chromosome 8 (Kirchgessner *et al.*, 1995; Sipley *et al.*, 1995). Lastly, *V3* hamster cells, transfected with yeast artificial chromosomes (YACs) containing the human DNA-PKcs gene, had regained normal DNA-PK activity, the ability to carry out V(D)J recombination and repair X-ray induced DNA dsbs (Blunt *et al.*, 1995). Subsequently, *scid* cells were shown to contain a single base pair alteration outside of the putative kinase domain of the

Table I-2: Genetic defects of cell lines belonging to the X-ray cross-complementing groups 4, 5, 6 and 7 (XRCC) (Adapted from Jeggo *et al.*, 1995; Weaver, 1996; Chu 1997). Presence of DNA-PKcs protein and Ku DNA-end binding activity for each cell type are indicated. XRCC5 and XRCC7 lack Ku80 and DNA-PKcs, respectively, while the gene mutated in XRCC4 encodes a novel protein with no significant amino acid sequence homology to any known proteins. The genetic defect for *XRCC6* has not been identified.

Complementation Group	**Mutant cell line**	**Ku DNA-End binding**	**DNA-PKcs Protein**	**Genetic Defect**
XRCC4	Hamster *XR1* Mouse *M10*	Yes	Yes	XRCC4 novel protein
XRCC5	Hamster *xrs* Hamster *VRV15B* Hamster *sxi-3* Hamster *sxi-2*	No No No No	Yes Yes Yes Yes	All Ku80
XRCC6	Hamster *sxi-1*	No	Yes	Unidentified
XRCC7	Mouse *scid* Hamster *V-3*	Yes Yes	No No	DNA-PKcs

DNA-PKcs gene, which results in a premature stop codon, yielding a C-terminal truncated protein that is unstable and present in extremely low abundance (Danska *et al.*, 1996; Blunt *et al.*, 1996). Therefore, the *scid* mouse may not represent a true DNA-PKcs knockout mouse model. Nonetheless, these elegant genetic and biochemical studies of the mutant cell lines belonging to the XRCC complementing groups 4, 5, 6 and 7 have established that DNA-PK is required in DNA dsb repair and V(D)J recombination pathways (reviewed in Jackson & Jeggo, 1995; Jeggo *et al*, 1995).

b) Lessons from other cell lines.

Studies from the *xrs* cells prompted researchers to ask whether or not other radiosensitive cell lines were defective for the components of DNA-PK. Several other hamster cell lines, an equine *scid* cell line, and one human cell line have since been shown to lack functional DNA-PK (summarized in Table I-3).

The hamster cell line *SX9* and equine *scid* cells are radiosensitive and unable to carry out V(D)J recombination in transient rearrangement assays (Fukumura *et al.*, 1998; Shin *et al.*, 1997). The mutation of the DNA-PKcs gene in *SX9* cells has been localized to a single T to C nucleotide transition in the coding region upstream of the putative PI-3 kinase domain, that causes a substitution of leucine 3191 to proline (Fukumura *et al.*, 1998). In equine *scid* cells, the mutation has been identified as a 5 bp deletion in the coding sequence that results in a truncated form of the DNA-PKcs protein that lacks 978 amino acids from its C-terminus (Shin *et al.*, 1997). In both cases, the mutation still allowed for expression of DNA-PKcs proteins, albeit at lower levels compared to wild type cells (Fukumura *et al.*, 1998; Shin *et al.*, 1997).

The human cell lines MO59J and MO59K were initially isolated from different portions of the same malignant glioma biopsy specimen (Allalunis-Turner *et al.*, 1993). Interestingly, only the MO59J cells are sensitive to ionizing radiation and lack detectable DNA-PKcs protein and DNA-PK activity (Lees-Miller *et al.*, 1995). Although the DNA-PKcs gene is present in MO59J cells (Lees-Miller *et al.*, 1995), the level of DNA-PKcs specific transcripts is reduced 16-64 fold, while the protein level is at least 200 times lower, when compared to MO59K cells (Kulesza and Lieber, 1998). Moreover, the ability of MO59J cells to perform V(D)J recombination is similar to that of the *scid* cells

Table I-3: Other cell lines and mouse models that are defective for DNA-PK components. The major phenotypes of each cell line or mouse model have been outlined. The formation of coding joint and signal joint are required during the process of V(D)J recombination (discussed in Chapter I.9).

DNA-PK component	**Cell line and mammalian source**	**Characterized deficiencies**	**Reference**
DNA-PKcs	*DNA-PKcs*$^{-/-}$ (knockout mouse)	DNA dsb repair, Coding joint formation	Gao *et al.*, 1998; Taccioli *et al.*, 1998
DNA-PKcs	MO59J (Human)	DNA dsb repair, Coding joint formation	Lees-Miller *et al.*, 1995; Kulesza and Lieber, 1998.
DNA-PKcs	*slip* (Knockout Mouse)	Thymic lymphoma	Jhappan *et al.*, 1997
DNA-PKcs	*SX9* (Mouse cell line)	Coding and Signal Joint formation, DNA dsb repair	Fukumura *et al.*, 1998
DNA-PKcs	*irs20* (Hamster cell line)	No detectable DNA-PK activity	Peterson *et al.*, 1997.
DNA-PKcs	*scid* (Equine)	Coding and Signal Joint formation, DNA dsb repair	Shin *et al.*, 1997
Ku 80	*Ku80*$^{-/-}$ (Knockout Mouse)	Coding and Signal Joint formation, DNA dsb repair, slow growth	Nussenzweig *et al.*, 1996
Ku 70	*Ku 70*$^{-/-}$ (Knockout Mouse)	Thymic lymphoma, slow growth	Li *et al.*, 1998

(Kulesza and Lieber, 1998). The precise defect(s) responsible for the low levels of DNA-PKcs transcripts in the MO59J cell line has not been determined.

c) Lessons from mouse knockout models

Mouse knockout models for Ku80 and Ku70 have been created (Nussenzweig *et al.*, 1996; Li *et al.*, 1998) (Table I-3). Furthermore, three mouse models carrying a complete inactivation of the DNA-PKcs gene have been independently generated (Jhappan *et al.*, 1997; Gao *et al.*, 1998; Taccioli *et al.*, 1998). One of these DNA-PKcs-deficient transgenic mouse models (called *slip* mouse) was generated by the fortuitous integration of several copies of a fragment of a yeast transgene into the 5' end of the DNA-PKcs gene (Jhappan *et al.*, 1997). Nonetheless, the phenotypes of these mouse knockouts reaffirmed the initial conclusions drawn from the studies of the XRCC complementing mutant cell lines. However, several other unexpected observations arose suggesting new roles for Ku80, Ku70 and DNA-PKcs.

Ku80$^{-/-}$ mice displayed a profound deficiency in V(D)J recombination and the development of both T and B lymphocytes was arrested at early progenitor stages. Although these mice were viable and could reproduce, they were 40-60% smaller than their littermates (*Ku80*$^{+/+}$ and *Ku80*$^{+/-}$) (Nussenzweig *et al.*, 1996). Studies with fibroblasts derived from *Ku80*$^{-/-}$ embryos (MEFs) revealed slower doubling times and faster entry into senescence compared to their wild type counterparts. Moreover, the *Ku80*$^{-/-}$ MEFs failed to re-enter the cell cycle following γ-irradiation induced arrest (Nussenzweig *et al.*, 1996). All of these observations suggest a role for Ku80 in the control of cellular growth.

Ku70$^{-/-}$ mice showed a similar reduction in size (approximately 60%) compared to their wild type littermates (Li *et al.*, 1998). The absence of *Ku70* also arrested the development of B-cells at an early progenitor stage, but did not block TCR gene rearrangement or the development of T-cells. Significantly, *Ku70*$^{-/-}$ mice developed fatal thymic and disseminated T-cell lymphomas by about 28 weeks of age. Cultured primary *Ku70*$^{-/-}$ mouse fibroblasts showed a higher frequency of spontaneous transformation compared to wild type mouse fibroblasts. These transformed *Ku70*$^{-/-}$ cells showed anchorage independent growth in a soft agar assay and were able to cause tumors in nude

mice. These *in vivo* and *in vitro* data demonstrated that inactivation of *Ku70* leads to neoplastic growth and suggests a novel role for Ku70 in tumor suppression (Li *et al.*, 1998).

Interestingly, of the three DNA-PKcs knockout mouse models reported to date, only the *slip* mice showed a propensity to develop T-cell lymphomas (Jhappan *et al.*, 1997). Unlike *scid* mice, which harbor a single non-sense mutation within the DNA-PKcs gene, and therefore may not represent a true genetic null mutation for DNA-PKcs, all three transgenic mouse models harbor insertions in the DNA-PKcs coding sequences. This resulted in no detectable DNA-PKcs transcripts, protein or DNA-PK activity (Jhappan *et al.*, 1997; Gao *et al.*, 1998; Taccioli *et al.*, 1998). Similar to the *scid* mice, these mice lacked mature B and T lymphocytes due to an absence of V(D)J rearrangement, but unlike the *Ku70*$^{-/-}$ or *Ku80*$^{-/-}$ mice, these mice grew normally. However, all of the mice homozygous for the *slip* mutation, developed thymic lymphoblastic lymphoma and died before the age of 20 to 24 weeks (Jhappan *et al.*, 1997). Although *scid* mice also had a tendency to develop lymphomas, it occurred only in about 15% of the population. Thus, thymic lymphomas in *slip* mice occurred with complete and greater penetrance (Jhappan *et al.*, 1997).

In summation, the initial studies with radiosensitive cell lines and the phenotypes of the mouse knockouts have unequivocally shown that DNA-PK is required for NHEJ events. In addition, functional DNA-PKcs and Ku70 are required to prevent neoplastic proliferation of developing T lymphocytes and both Ku70 and Ku80 are required for normal development in mice. Taken together these phenotypes suggest that DNA-PK may be involved in regulating the cell cycle during development. Further studies within these cell lines, as well as in yeast, have assigned possible roles for DNA-PK in the NHEJ pathway during DNA dsb repair and V(D)J recombination. However, as it shall be revealed in the following discussion, outstanding concerns and questions remain.

I.9: DNA-PK and NHEJ - Molecular mechanisms.

a) DNA-PK and V(D)J recombination

The process of V(D)J recombination involves the rearrangement of three distinct gene segments called variable (V), joining (J) and diversity (D) elements, which are

located at distinct loci, into one contiguous exon to encode for the Ig or TCR protein (reviewed in Lewis, 1994). One of the V elements can join with any J or D element, providing a mechanism to generate diversity. Each V, D, or J element, generally termed as a coding sequence, is flanked by a recombination signal sequence (RSS). The rearrangement process is initiated by the creation of a single nick at the border of the RSS and its adjacent coding sequence by RAG1 and RAG2, to expose a 3'-hydroxyl group on the side of the coding sequence (*Step 1*, Figure I-3) (reviewed in Grawunder *et al.*, 1998; Roth and Craig, 1998). The efficiency of this cleavage step is enhanced by the presence of HMG1 and HMG2, *in vitro* (van Gent *et al.*, 1997). The 3'-hydroxyl group then undergoes a nucleophilic attack on the opposite DNA strand of the coding sequence to form a coding hairpin end and a RSS blunt end (*Step 2*, Figure I-3). It has been proposed that RAG1 and RAG2, and possibly the HMG proteins, form a synaptic protein-DNA complex after the cleavage step that keeps the ends of coding and RSS fragments in close contact for the proper rejoining events (Grawunder and Lieber, 1997; Melek *et al.*, 1998). The RRS signal fragment with the blunt ends will be joined to yield precise head-to-head signal joints. The closed hairpin coding end will be nicked and processed to form a coding joint that is frequently imprecise, involving deletions of 10 bp or less, as well as insertions of up to three nucleotides (*Step 3*, Figure I-3). The insertion of additional nucleotides, catalyzed by the enzyme terminal deoxynucleotidyl transferase (TdT), adds an additional level of diversity. The opening of the hairpin coding end is likely catalyzed by RAG1 and RAG2, although there is evidence that another protein of the NHEJ apparatus (to be discussed later) is involved (Melek *et al.*, 1998; Paull and Gellert, 1998). Following the opening of the hairpin coding end, the exposed ds ends of the coding and RSS signal sequences are repaired by DNA-PK and the NHEJ apparatus.

The functions of DNA-PKcs and Ku in NHEJ during V(D)J recombination have been inferred from studies in cell lines deficient for DNA-PK. Unjoined hairpin coding and signal ends accumulate in *Ku80*$^{-/-}$ mice thymocytes (Bogue *et al.*, 1997), whereas only coding hairpin ends accumulate in *scid* and *DNA-PKcs*$^{-/-}$ mice thymocytes (Roth *et al.*, 1992; Gao *et al.*, 1998; Taccioli *et al.*, 1998). Further, mouse embryonic stem cells inactivated for the *Ku70* gene were not able to join signal and coding ends in transient recombination assays (Gu *et al.*, 1997). These data suggest that DNA-PKcs is required

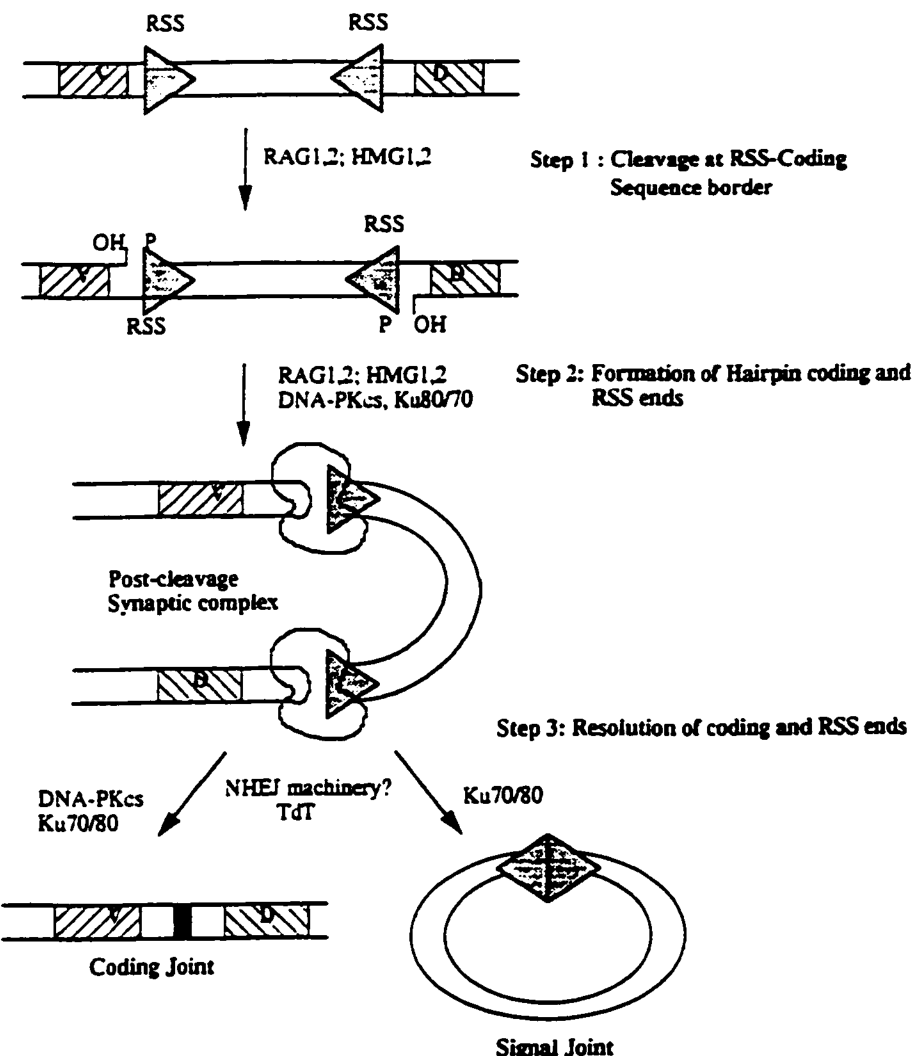

Figure I-3: Schematic representation of the process of V(D)J recombination. Steps outlined indicate the possible role of DNA-PK in V(D)J recombination (Adapted from Lieber *et al.*, 1997; Grawunder *et al.*, 1998). *Step 1 - Cleavage at RSS-Coding sequence border*. RAG1 and RAG2 with the aid of HMG1 and HMG2 cleaves at RSS sites (indicated by filled-in triangles) adjacent to Variable (V) or Diversity (D) coding sequences exposing 3'OH and 5'PO_4 groups. *Step 2 - Formation of hairpin coding end and RSS blunt end.* The 3'OH group undergoes a nucleophilic attack on the opposite strand of the coding sequence to create a hairpin structure and RSS blunt ends. A resulting post-cleavage synaptic complex containing RAG 1 and 2, HMG 1 and 2 is required to maintain the proper configuration of the ends to allow efficient joining. *Step 3 - Resolution of coding and RSS ends*. DNA-PK, components of the NHEJ pathway and TdT, enter and resolve the two coding and signal ends. Formation of the coding joint and signal joint reflect a successful V(D)J rearrangement process.

for the formation of coding joints, while Ku80 and Ku70 are required for the formation of coding and signal joints.

Lending support to this hypothesis, is the observation that MO59J cells are capable of joining signal ends but not coding ends in transient recombination assays (Kulesza and Lieber, 1998). Similar results have been obtained with mouse embryo fibroblasts (MEFs) from *DNA-PKcs*$^{-/-}$ mice (Gao *et al.*, 1998; Taccioli *et al.*, 1998). However, studies with two other DNA-PKcs-deficient cell lines, *SX9* cells and equine *scid* cells, have shown that these cells were unable to form coding and signal joints (Fukumura *et al.*, 1998; Shin *et al.*, 1997). Interestingly, DNA-PK enzyme activity in *SX9* cells is decreased by 50%, even though protein and mRNA levels were similar to wild type parental cells (Peterson *et al.*, 1997; Fukumura *et al.*, 1998). This suggests that DNA-PK kinase activity may be required for the proper resolution of the signal and hairpin coding ends. On the other hand, it was suggested that the genetic mutations in the *SX9* and equine *scid* resulted in DNA-PKcs gene products that are being expressed at a level that may act as "dominant-negative inhibitors" by interacting with Ku70 and Ku80, thereby inhibiting their activity during signal joint formation (Kulesza and Lieber, 1998; Gao *et al.*, 1998; Taccioli *et al.*, 1998). In MO59J cells and MEFs from *DNA-PKcs*$^{-/-}$ mice, DNA-PKcs is not present at a sufficient level to impose this possible dominant negative effect on Ku80 and Ku70 (Kulesza and Lieber, 1998).

To date, there is very little biochemical data to precisely define the role of DNA-PK in resolving coding and signal ends. Antibodies to either DNA-PKcs, Ku70 or Ku80 precipitate signal and coding ends from an *in vitro* assay utilizing recombination active crude cell extracts and a ^{32}P-labeled DNA rearrangement substrate which contains RSSs (Agrawal and Schatz, 1997). Coding and signal ends were also precipitated by antibodies to RAG1 and HMG1. These results indicate that DNA-PK likely associates with HMG1/2 and RAG1/2 in the post-cleavage synaptic complex during the formation of the signal and coding joint (Agrawal and Schatz, 1997). EMSA studies indicate that DNA-PK can bind to a 68 bp DNA construct with two hairpin ends; however, DNA-PK kinase activity is not stimulated (Smider *et al.*, 1998). DNA-PK also associates with the same 68 bp DNA construct with a single nick, but kinase activity is not stimulated. These DNA constructs represent intermediates believed to be required for joining hairpin

coding ends, therefore suggesting that DNA-PK kinase activity may not be necessary for this process. Taken together, these data also suggest that DNA-PK may only play a structural role, in association with RAG1/2 and HMG1/2, to maintain broken DNA ends in proper configuration for end-joining. Results from further studies on NHEJ in mammalian and yeast systems lend support to this notion.

b) Model for NHEJ

There are two approaches that are frequently used to study DNA dsb repair *in vivo*. In the first approach, plasmids containing a marker gene are linearized with restriction enzymes and transfected into cells; the success and efficiency of repair is monitored by the type and number of rejoined plasmids recovered. In the second approach, chromosomal DNA breaks are introduced in the cell either by inducing expression of rare cutting restriction enzymes or by exposing cells to ionizing radiation or DNA damaging drugs; success of dsb repair in this case is monitored by the survival rate of the cells (reviewed in Chu, 1997; Lieber *et al.*, 1997). Based on the results of these studies, a three-step model for NHEJ has been proposed (Figure I-4) (Philips & Morgan, 1994; Lieber *et al.*, 1997; Chu, 1997). First, the two broken ends must be "brought together" and maintained in synapses (*Step 1*, Figure I-4). Second, the ends must then be reconfigured biochemically into a ligatable form (*Step 2*, Figure I-4). This is thought to entail nucleolytic activity that removes several nucleotides (1-6) from each broken end until a region of complementary sequences is reached. Once a region of "microhomology" has been established, excess single-stranded DNA extensions are trimmed by nucleases. Finally, DNA polymerases enter to fill any gaps and DNA Ligases seal the nicks to produce the final repaired DNA joint (*Step 3*, Figure I-4). It is likely that the joining of coding and signal ends during V(D)J recombination follows a similar pathway (reviewed in Chu, 1997; Grawunder *et al.*, 1998; Critchlow and Jackson, 1998).

c) NHEJ - Studies in mammalian systems

Based on genetic evidence, it was established that DNA-PK is required for NHEJ during DNA dsb repair. Further biochemical data to precisely define a role for DNA-PK in NHEJ is scarce, but these results are, at the very least, insightful. *In vivo*, *scid* cells

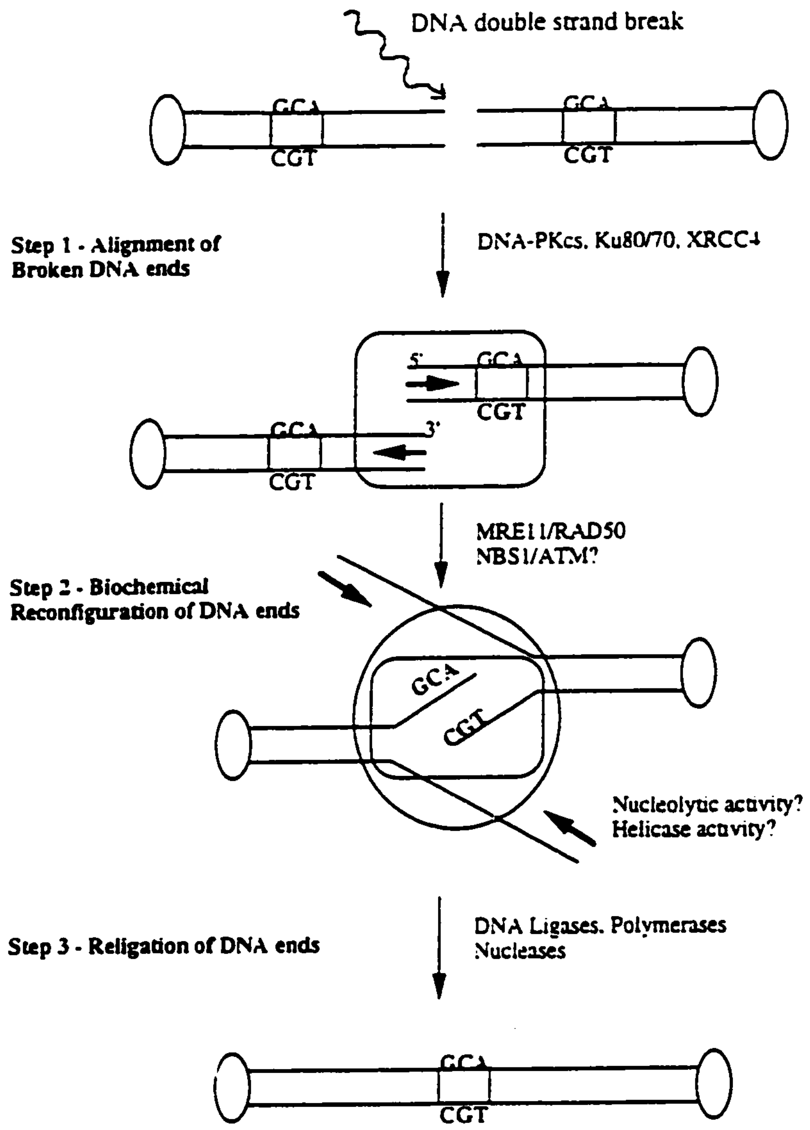

Figure I-4: Model for the Non-homologous End-Joining process in mammals (Adapted from Lieber *et al.*, 1997; Chu 1997; Dynan and Yoo, 1998). DNA double strand breaks can occur during exposure of DNA to oxygen radicals. Once the ends of DNA become exposed, the following steps may occur to ensure proper repair of the damaged site. *Step 1 - Alignment of broken DNA ends*: The DNA-PK trimeric enzyme complex, with the aid of XRCC4, may initially bind to the dsDNA ends to maintain the exposed ends in a manner that is "structurally favorable" for subsequent steps. *Step 2 - Biochemical reconfiguration of DNA ends*: The Mre11/Rad50 complex may be recruited by either ATM or Nibrin/NBS1 to the site of the DNA dsb. Mre11/Rad50 complex may expose sites of homology between the two DNA strands via the nucleolytic activity of Mre11. Alternatively, the helicase activity of Ku may be responsible for unwinding the DNA to expose these complementary sequences. *Step 3 - Religation of DNA ends*: Once the region of "micro-homology" is established, DNA-ligases, polymerases and other nucleases may enter to process and rejoin the broken ends.

can repair transfected plasmid DNA containing staggered or blunt ends equally well compared to wild type parental cells. Conversely, the repair rate of γ-irradiated DNA chromosomal breaks is reduced 3 to 5 fold compared to wild parental cells (Chang *et al.*, 1993). These data suggest that the mutation in DNA-PKcs in *scid* affects repair of DNA at the chromosomal level. To date, similar DNA repair studies have not been done in other cells deficient for DNA-PKcs, such as cells from DNA-PKcs knockout mice, or the *SX9* or MO59J cell lines.

Ku80-deficient cell lines also display low rates of repair of radiation induced dsbs (Taccioli *et al.*, 1994). These cells can also repair transfected linearized plasmids, but unlike *scid* cells, the recovered rejoined plasmids are imprecise and contain large deletions (Liang and Jasin, 1996). This suggests a role for Ku80 in protecting DNA ends from degradation during repair. In a more detailed study, Jasin and co-workers created a vector which had a selectable marker (neo) that contained a restriction site for a rare restriction enzyme, I-SceI (Liang *et al.*, 1996). The restriction site was engineered such that, when it is cut, the wild type marker gene is reestablished only if the NHEJ event occurs with deletion of the ends to a region of "microhomology" of 4 bp. This construct was stably integrated into *xrs-6* cells (*Ku80* minus) and a wild type parental strain (CHO-K) to assess the efficiency of NHEJ in context of the chromatin. *Xrs-6* cells were 300 times less effective in producing the wild type marker compared to the wild type parental cells (Liang *et al.*, 1996). These data suggest that Ku80 is required for NHEJ, but the precise role of Ku80 remains uncertain.

Further studies have suggested possible roles for Ku and have introduced other proteins in NHEJ. The final step of NHEJ involves the intermolecular ligation of the DNA ends (Figure I-4). In mammals, there are four types of DNA Ligases, termed DNA Ligase I-IV. While all seem to be proficient in sealing DNA nicks, DNA Ligase I ligates blunt ends very efficiently, while DNA Ligases III and IV prefer complementary overhanging ends of 4 bp or more for efficient joining (Grawunder *et al.*, 1997; Wilson *et al.*, 1997). The ability of Ku to stimulate the joining activity of DNA Ligase I, III, and IV, has been examined in an *in vitro* ligation assay using DNA substrates with either blunt, 2 bp, or 4 bp complementary overhanging ends (Ramsden and Gellert, 1998). The greatest fold stimulation of activity was seen with DNA Ligase I and substrates with

either blunt or 2 bp overhanging ends. DNA Ligases III and IV showed no activity towards blunt ended substrates even in the presence of Ku; on the other hand, their efficiency at joining substrates with overhanging complementary ends, increased 30 fold in the presence of Ku. DNA Ligase I has been previously demonstrated to stimulate V(D)J recombination in a cell free recombination assay system (Ramsden *et al.*, 1997). These data are consistent with a role for Ku in maintaining ends of DNA in close proximity for end-joining.

As discussed earlier (section I.8), a novel gene product XRCC4 has been shown to correct V(D)J recombination defects and restore X-ray resistance in the mutant cell line *XR-1* (Li *et al.*, 1995). Recently, baculovirus expressed XRCC4 has been shown to facilitate Ku binding to DNA and to promote the assembly of the DNA-PK complex on DNA (Leber *et al.*, 1998). DNA-PK also phosphorylates recombinant XRCC4 *in vitro* (Leber *et al.*, 1998). Interaction between XRCC4 and DNA Ligase IV has been shown by co-immunoprecipitation, gel-filtration chromatography and yeast two hybrid assays (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997). This association led to a 5 to 7 fold increase in DNA Ligase IV joining activity for plasmids with complementary overhanging ends *in vitro* (Grawunder *et al.* 1997). It has been reported that DNA-PK can be detected in immunoprecipitation complexes with XRCC4 and DNA Ligase IV, albeit in non-stoichiometric amounts (Critchlow *et al.*, 1997). It is likely that Ku, possibly in complex with DNA-PKcs, facilitates DNA Ligase activity by bridging the DNA ends at regions of "microhomology" (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997). This notion is consistent with the work of Bliss and Lane (1997), which shows that Ku can transfer between two molecules of DNA with complementary ends. XRCC4 may act to stabilize the interaction between DNA-PK and DNA Ligases to enhance the ligation process (Critchlow and Jackson, 1998).

d) NHEJ - Further lessons from the budding yeast

Further insights into the mechanisms of NHEJ can be drawn from studies on the budding yeast. When the yeast homologue of either Ku70 (*YKU70* or *HDF1*) or Ku80 (*YKU80* or *HDF2*) is individually inactivated, yeast cells do not become sensitive to ionizing radiation (IR) or to other DNA damaging agents such as MMS (Methyl

Methanesulfonate) (Siede *et al.*, 1996). However, when the homologous recombination apparatus was rendered inoperative by mutations to *RAD52*, inactivation of *YKU70* or *YKU80* led to significantly enhanced sensitivity to IR and MMS (Siede *et al.*, 1996; Boulton and Jackson, 1996a,b). Yeast *yku70/rad52* double mutant strains carrying a third mutation to the yeast homologue of DNA Ligase IV (*DNL4*), showed no further increase in radiosensitivity to IR (Wilson *et al.*, 1997). This indicates that both *YKU70* and *DNL4* gene products act in the same pathway. In plasmid rejoining assays, linearized plasmids transfected into *rad52* mutant cells were ligated with small deletions (2-16 bp) at sites corresponding to short direct repeat elements of 1-6 bp. On the other hand, rejoined plasmids recovered from *dnl4/rad52*, *yku70/rad52*, *yku80/rad52* double mutant strains, contained large deletions (6-95 bp) and were ligated at sites with short direct repeat sequences (2-3 bp) (Wilson *et al.*, 1997; Boulton and Jackson, 1996a,b; 1998). These data suggest that in yeast, *YKU70*, *YKU80* and *DNL4* are required for an efficient microhomology-directed end-joining DNA repair pathway that functions in the absence of an intact *RAD52*-dependent homologous recombination system.

Other genes that have been identified via genetic screens using homologous recombination deficient yeast strains, include *MRE11*, *XRS2*, *RAD50*, and the *SIR2*, *3*, and *4* genes (reviewed in Weaver, 1998; Critchlow and Jackson, 1998). The discovery of *SIR* genes in this screen was surprising because they were known to be involved in telomere directed transcription repression in yeast (Tsukamoto *et al.*, 1998), implying that telomere maintenance and NHEJ in yeast utilize similar gene products (to be discussed later). Deletion of any one of these genes in a *rad52* mutant leads to an increased sensitivity of yeast cells to IR (reviewed in Weaver, 1998). Human homologues of *MRE11, RAD50* and *XRS2* have been identified, and recent studies have shown them to be vital players in NHEJ (Petrini, *et al.*, 1995; Dolganov, *et al.*, 1996; Carney *et al.*, 1998).

In human cells, hMre11 and hRad50 associate with one another in a multiprotein complex and, in response to IR, both proteins are present in discrete nuclear foci that correspond specifically to the sites of DNA damage (Dolganov *et al.*, 1996; Maser *et al.*, 1997; Nelms *et al.*, 1998). This radiation induced co-localization of hMre11 and hRad50 is reduced in SV40 transformed A-T (Ataxia Telangiectasia) cell lines, but not in MO59J

or MO59K cells, suggesting that ATM and not DNA-PKcs, may have a role in recruiting hMre11/Rad50 to the sites of DNA damage (Maser *et al.*, 1997). A similar reduction in radiation induced co-localization of hMre11 and hRad50 is seen in Nijmegen breakage syndrome (NBS) cells (Carney *et al.*, 1998). NBS is an autosomal recessive disorder characterized by increased incidence in cancer, cell cycle checkpoint defects and ionizing radiation sensitivity (reviewed in Shiloh, 1997). The gene mutated in NBS has recently been shown to encode a novel 95 kDa protein called p95/NBS1 or nibrin, which has limited amino acid similarity to yeast Xrs2p (Varon *et al.*, 1998; Carney *et al.*, 1998). Human Mre11, Rad50 and p95/NBS1/nibrin co-elute from a gel-filtration column and co-immunoprecipitate, suggesting that the interaction of Mre11/Rad50 with p95/nibrin may be required for co-localization of Mre11/Rad50 in the nucleus following exposure to IR (Carney *et al.*, 1998).

Recent work of Paull and Gellert (1998) has shed light on the possible role of hMre11 and hRad50 in NHEJ. They showed that baculovirus expressed hMre11 has 3' to 5' exonucleolytic activity, which is enhanced in the presence of the hRad50 (Paull and Gellert, 1998). Furthermore, hMre11 and hRad50 together with DNA Ligase I, can rejoin plasmids with blunt or mismatched 5' overhanging ends *in vitro*. The repaired products display regions of 1-5 bp microhomologies at the joined ends, with deletions between 13-73 bp. These deletions are larger than rejoined plasmids recovered from plasmid rejoining assays carried out *in vivo* (Liang and Jasin, 1996). The authors attributed this observation to the absence of Ku, in that *in vivo*, Ku may have a role in limiting the number of nucleotides removed (Paull and Gellert, 1998). These data are consistent with the observation that rejoined transfected plasmids recovered from Ku80-deficient cells (*xrs6*) contained very large nucleotide deletions (Liang and Jasin, 1996). However, these data may not reflect repair in the physiological context of chromatin. Signal and hairpin coding ends have been detected in *Ku80*$^{-/-}$ mice thymocytes suggesting that Ku may not be solely involved in the protection of DNA ends during NHEJ (Bogue *et al*, 1997). Nevertheless, these data suggest that MRE11 may catalyze the removal of nucleotides in the 3' to 5' direction to reveal homologous sequences during NHEJ.

Paull and Gellert (1998) further showed that Mre11 exhibits endonuclease activity on hairpin loops, suggesting that it may have a role in opening hairpin coding ends

generated by the RAG1 and RAG2 proteins during V(D)J recombination. This further suggests that the NHEJ apparatus, which includes DNA-PK, is responsible for joining the coding and signal ends generated during V(D)J recombination. The importance of MRE11 in mammalian cells is reiterated in the fact that MRE11 is essential for viability in murine embryonic stem cell line (Xiao and Weaver, 1997).

e) Synopsis of the role for DNA-PK in NHEJ

The molecular apparatus and mechanism defining the proposed NHEJ pathway responsible for joining DNA ends exposed during DNA dsbs or V(D)J recombination are slowly being revealed from studies in mammalian and yeast systems (reviewed in Featherstone and Jackson, 1998; Weaver, 1998; Critchlow and Jackson, 1998). Although direct biochemical evidence is still lacking, models for DNA-PK activity in NHEJ can be proposed (Figure I-4). It is likely that Ku70/80 will bind initially to the ends of broken DNA and recruit DNA-PKcs to form a trimeric complex that keeps the broken ends synapsed and aligned (*Step 1*, Figure I-4). XRCC4 may have a role in potentiating the association of DNA-PK to the broken ends. Once the trimeric DNA-PK complex takes up a position at the broken DNA ends, Ku may then translocate along the DNA and, through its helicase activity, unwind the DNA to scan for a region of direct repeat sequence. Alternatively, hMre11/hRad50 may enter the damaged site and through the 3'-5' nucleolytic activity of MRE11, uncover the region of homology (*Step 2*, Figure I-4). In considering recent data, NBS1 and/or ATM are likely to be involved in localizing hMre11/hRad50 to the site of the DNA lesion (Maser *et al.*, 1997; Carney *et al.*, 1998); whether or not these proteins co-operate with DNA-PK during NHEJ remains to be determined. The resulting "ssDNA" flaps are degraded, DNA polymerases enter to fill the gaps, and DNA Ligases in association with Ku and XRCC4 join the ends (*Step 3*, Figure I-4). Several questions remain in regards to the role of DNA-PK in NHEJ. Is DNA-PK kinase activity required during NHEJ? Does DNA-PK associate and recruit other proteins to the sites of DNA dsbs? As mentioned earlier, the genes required for NHEJ are also involved in telomere maintenance in yeast. Studies into telomere maintenance (to be discussed later) have revealed further insights into the pathway of NHEJ.

I.10: DNA-PK and cellular response to DNA damage

In addition to being involved in the actual DNA dsb repair process itself, there is evidence to suggest that DNA-PK may play a role in cellular response pathways to DNA damage (reviewed in Hoekstra, 1997; Wang, 1998). Proliferating cells can delay and/or arrest cell cycle progression in response to genotoxic stress, in order to avoid the replication or the segregation of damaged DNA. This delay or arrest at G1/S (replication) or G2/M (segregation) allows for proper repair of the genome. If the damage is deemed irreparable, cells in multicellular organisms can activate programmed cell death or apoptosis. This allows the organism to remove cells that carry too many genetic lesions, of which some may be tumorigenic. Therefore, cells must have the ability to sense damaged DNA and simultaneously signal to proteins required for cell cycle control and DNA repair in order to maintain the fidelity of the genome. Evidence to suggest that DNA-PK is involved in this pathway surfaced from studies on three proteins: p53, c-Abl and poly ADP-ribose polymerase (PARP).

a) DNA-PK and p53

The tumor suppressor protein p53, is a transcription factor whose protein levels and transcriptional activity increase upon DNA damage. Increases in protein levels and in transcriptional activity are believed to be consequences of post-translational mechanisms and are required for both cell cycle arrest and apoptosis, depending on the cell type examined (reviewed in Ko and Prives, 1996; Gottlieb and Oren, 1996). Genes that are transcriptionally activated by p53 include the cyclin kinase inhibitor p21, which is one of the proteins responsible for cell cycle arrest. Thus, understanding the regulation of p53 in response to DNA damage is central to our understanding of cellular DNA damage response pathways.

Initial evidence for a role for DNA-PK in the DNA damage response pathway came from studies on the DNA-PK phosphorylation sites on p53. DNA-PK phosphorylates the amino-terminus of p53 on Ser15 and Ser37 *in vitro* (Lees-Miller *et al.*, 1992). Mutation of Ser15 in p53 reduces (by about 50%) the ability of p53 to suppress growth compared to wild type p53, suggesting that p53 activity may be regulated by DNA-PK phosphorylation *in vivo* (Fiscella *et al.*, 1993). However, further

studies with cells from *scid* mice have demonstrated that DNA-PK is not required for p53 function *in vivo*. *Scid* cells show normal G1/S cell cycle arrest that coincides with the accumulation of p53 in response to DNA-damage induced by γ-irradiation, MMS or micro-injected linearized plasmids (Fried *et al.*, 1996; Huang *et al.*, 1996; Rathmell *et al.*, 1997). In each case, the transcriptional activity of p53 was upregulated as indicated by the increase of p21 transcripts. Further, Guidos and colleagues reported that $p53^{-/-}$*/scid* double mutant mice develop disseminated pro-B or immature T-cell lymphoma/leukemia by 7-12 weeks of age (Guidos *et al.*, 1996). The authors interpreted this result to mean that in *scid* mice, the p53 mediated DNA damage checkpoint remains functional and protects cells against DNA dsbs generated from inefficient V(D)J recombination events. Bypassing this checkpoint in the double mutant $p53^{-/-}$*/scid* mice, allowed *scid* lymphocyte precursors to harbor broken V(D)J coding ends, leading to the accumulation of aneuploid cells and the eventual development of tumors (Guidos *et al.*, 1996). Collectively, these data are consistent with the idea that DNA-PKcs is not required for p53 mediated cell cycle attenuation in response to DNA damage. On the other hand, these data suggest the possible existence of another parallel pathway which compensates for the absence of DNA-PKcs.

These studies with *scid* mice were done primarily with *scid* mouse embryo fibroblasts (MEFs), prior to the identification of the precise genetic defect in the *scid* mutation (Danska *et al.*, 1996; Blunt *et al.*, 1996). Since the mutation in the DNA-PKcs gene in *scid* is not a complete null mutation, *scid* cells may contain residual DNA-PK activity (Danska *et al.*, 1996). It has been reported recently that *scid* MEFs do contain detectable DNA-PK activity, albeit lower than wild type BALB MEFs (Woo *et al.*, 1998). In the same study, a transformed *scid* cell line called SCGR11 was shown to exhibit little to no detectable DNA-PK activity (Woo *et al.*, 1998). Using SCGR11 *scid* cells, Lee and colleagues reported that although p53 accumulated in response to DNA damaging agents (γ-irradiation, hydrogen peroxide and MMS), upregulation of p21 transcripts was not seen (Woo *et al.*, 1998). Absence of p21 upregulation coincided with the lack of p53 binding to its consensus DNA binding sequence. The human glioma cell lines MO59J and MO59K were also tested in this study. In EMSA studies, binding of *in vitro* translated p53 to its consensus sequence was seen only when p53 was incubated

with nuclear extracts from γ-irradiated MO59K cells and not with nuclear extracts from γ-irradiated MO59J cells. Addition of purified DNA-PKcs to the nuclear extracts from γ-irradiated MO59J cells, restored p53 binding activity. Further, addition of a DNA-PK substrate peptide abolished p53 binding activity, while the presence of a non-DNA-PK substrate peptide had no effect, suggesting that DNA-PK kinase activity may be required for p53 DNA binding activity. Taken together, the findings of Lee and coworkers show that DNA-PK may stimulate p53 DNA binding activity following DNA damage. Moreover, p53 binding was seen only with both DNA-PKcs and irradiated nuclear extracts suggesting that additional nuclear factor(s) that become activated during DNA damage are required. These results place DNA-PK upstream of p53 which contradicts earlier observations. The difference could be attributed to the cell lines used and to the unpredictable phenotype of the *scid* mutation. This issue could be resolved by performing similar studies using the cells from DNA-PKcs knockout mice.

Further data to support the hypothesis that DNA-PK may act upstream of p53 comes from elegant biochemical studies of Prives and coworkers (Shieh *et al.*, 1997). By using antibodies that specifically recognize phosphorylated Ser15 of human p53, it was demonstrated that human p53 is phosphorylated at Ser15 in response to γ-irradiation. *In vitro*, recombinant p53 that was phosphorylated by DNA-PK on Ser15 and Ser37, showed a decreased ability to interact with human baculovirus expressed MDM2. It has been previously shown that MDM2 interacts with p53 and targets p53 for destruction *in vivo* (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). In extracts from γ-irradiated cells, the amount of p53 co-immunoprecipitated with MDM2 decreased; coincidentally, p53 in these immune complexes showed increased phosphorylation of Ser15. Taken together, these data suggest that phosphorylation of p53 at Ser15 alleviates its interaction with MDM2, preventing p53 destruction, leading to the accumulation of p53 following DNA damage (Shieh *et al.*, 1997). Since DNA-PK phosphorylates p53 at these sites *in vitro*, it may also be responsible for the accumulation of p53 in response to DNA damage, *in vivo*.

It was recently shown that, another member of the PI-3 related kinase family, ATM, also phosphorylates Ser15 on p53 *in vitro*. Phosphorylation of Ser15 on p53 is enhanced with ATM immunoprecipitated from cells treated with the radiomimetic drug neocarzinostatin and ionizing radiation (Banin *et al.*, 1998; Canman *et al.*, 1998).

However, A-T cells still show accumulation of p53, albeit with delayed kinetics, in response to DNA-damage (Canman *et al.*, 1994). Whether or not DNA-PK also phosphorylates p53 in response to DNA damage *in vivo* remains to be determined. The best avenue to pursue this question would be to look at p53 phosphorylation status in irradiated cells from DNA-PKcs-deficient mice. It is very likely that DNA-PK and ATM act in parallel pathways that signal to p53. For instance, DNA-PK may be responsible for the delayed p53-dependent G1 arrest in response to DNA-damage seen in A-T cells (Canman *et al.*, 1994), while ATM induces normal p53 dependent G1 arrest seen in *scid* cells. Definitive resolution of this possibility awaits the creation of a cell line in which both DNA-PKcs and ATM are inactivated.

b) DNA-PK and c-Abl

Another protein that could be targeted by DNA-PK in response to DNA damage is the tyrosine kinase encoded by the proto-oncogene *c-abl*. The kinase activity of c-Abl towards the CTD of RNA polymerase II is stimulated by IR or MMS. Phosphorylation of the CTD has been linked to promoter clearance and transcription elongation, and it has been proposed that phosphorylation of the CTD by c-Abl may regulate gene expression in response to DNA damage (Liu *et al.*, 1996). DNA-PKcs and c-Abl co-immunoprecipitate and this association is enhanced in response to ionizing radiation and MMS treatment (Kharbanda *et al.*, 1997). Ku also co-immunoprecipitates with DNA-PKcs and c-Abl but only in cell extracts isolated after DNA damage. DNA-PK phosphorylates and activates recombinant c-Abl to phosphorylate another of its substrates, GST-Crk (an SH3 binding protein) *in vitro*. c-Abl isolated from γ-irradiated DNA-PKcs$^{+/+}$ cells (wild type CB17) show a 2.9 fold stimulation towards GST-Crk compared to 1.5 fold stimulation of c-Abl activity isolated from irradiated *scid* cells (DNA-PKcs$^{-/-}$). Interestingly, recombinant c-Abl is able to phosphorylate DNA-PKcs on a Tyr residue causing the dissociation of DNA-PKcs from Ku (Kharbanda *et al.*, 1997; Jin *et al.* 1997). It has been proposed that dissociation of DNA-PKcs from Ku is the mechanism of inactivation of DNA-PKcs kinase activity following DNA-PK autophosphorylation (Chan and Lees-Miller, 1996). The physiological relevance of these observations has yet to be explored, although it can be envisioned that activation of c-Abl

by DNA-PK in response to DNA damage may serve as an auto-regulatory mechanism for DNA-PK activity. Moreover, DNA-PK may control transcription of certain genes by modulating c-Abl activity.

The exact role of c-Abl in radiation induced DNA damage response remains to be defined. Fibroblasts derived from *c-abl* mutant mice are not hypersensitive to IR, nor do they exhibit any detectable defects in IR-induced cell cycle checkpoints (Liu *et al.*, 1996). It is therefore likely that c-Abl is part of one DNA lesion response pathway mediated by DNA-PK that may not be the primary pathway leading to cell cycle arrest. One possibility is that this pathway may lead to the expression of certain genes required for DNA repair. The exact consequences of these interactions within this pathway remain to be elucidated.

b) DNA-PK and PARP

A protein that may cooperate with DNA-PK in response to DNA damage is the nuclear enzyme poly ADP-ribose polymerase (PARP). PARP is stimulated by discontinuities of DNA structure such as nicks, gaps and double strand breaks to catalyze the formation of ADP ribose polymers onto protein acceptors using NADH (nicotinamide adenine dinucleotide) as substrate (reviewed in Lindahl *et al.*, 1995). Some of the protein acceptors identified to date include PARP itself, DNA polymerase δ, ε, DNA Ligase I, and more recently, DNA-PKcs (reviewed in Weaver, 1996; Ruscetti *et al.*, 1998).

In response to DNA damage, PARP automodifies itself to create a long branched polymer of ADP-ribose units. Inhibitors of this activity have been shown to sensitize cells to DNA damaging reagents. Furthermore, cells expressing a dominant negative form of PARP, are hypersensitive to ionizing radiation and DNA alkylating agents (Kupper *et al.*, 1995; Schreiber *et al.*, 1995). It has been hypothesized that the production of ADP-ribose polymers creates a highly negatively charged molecule that mimics the DNA phosphate backbone and recruits DNA repair enzymes (reviewed in de Murcia and Menissier de Murcia, 1994; Lindahl *et al.*, 1995). Alternatively, the ribose polymer may attract positively charged histones and thus remodel the chromatin, to allow access for the DNA repair machinery. Furthermore, the presence of the ribose polymer may also inhibit certain cellular events, such as recombination between two homologous DNA sequences,

and promote DNA repair by NHEJ (Lindahl *et al.*, 1995; Jackson, 1996). The phenotypes observed for the PARP knockout mice are consistent with this speculation (Wang *et al.*, 1995; 1997).

PARP knock out mice are healthy, fertile and show unaltered development of T and B lymphocytes (Wang *et al.*, 1995). They show normal DNA excision repair (NER) activities as they are insensitive to UV irradiation and alkylating agents. However, *PARP*$^{-/-}$ mutant cells exhibit elevated levels of spontaneous sister chromatid exchange (Wang *et al.*, 1997). This reflects a higher rate of rejoining of chromosomal breaks by homologous recombination, suggesting that PARP is required to modulate recombination events (Wang *et al.*, 1997). To investigate the possibility of a functional interaction between DNA-PK and PARP, *PARP*$^{-/-}$ mice have been bred with *scid* mice (Morrison *et al.*, 1997). The double mutant *PARP*$^{-/-}$*/scid* progeny are fertile, but weigh 70% less than wild type heterozygous and hemizygous littermates. They have a mature T-cell repertoire which indicated that the *scid* block was bypassed. However, B-cell development did not progress past the progenitor stage, similar to the phenotype seen in *scid* mice. Furthermore, these double mutant mice developed T-cell lymphoma after 4 to 6 weeks. This phenotype resembles that of the *p53*$^{-/-}$*/scid* double mutant mice, except no B-cell lymphomas are detected (Guidos *et al.*, 1996). One plausible explanation for these phenotypes is that PARP normally acts to repress homologous recombination (Lindahl *et al.*, 1995; Morrison *et al.*, 1997); the absence of PARP stimulates some sort of rearrangement event via homologous recombination, thereby bypassing the *scid* block. However, in the *PARP/scid* double mutant mice, this process becomes deregulated resulting in chromosomal aberrations that eventually lead to cellular oncogenesis (Morrison *et al.*, 1997).

PARP has been shown to associate with DNA-PKcs and Ku *in vitro*, and PARP can ADP-ribosylate DNA-PKcs to stimulate DNA-PK activity towards p53 and RPA (Ruscetti *et al.*, 1998). This stimulation can be blocked by the specific PARP inhibitor, dihydroxyisoquinoline (DHQ). Intriguingly, PARP was also found to stimulate Ku independent DNA-PKcs activity towards a novel substrate hnRNP-U (hetero-nuclear Ribonucleoprotein-U). DNA-PK also phosphorylates PARP but this event does not affect the automodification or DNA binding activity of PARP. These *in vivo* and *in vitro*

data suggest that PARP and DNA-PK functionally cooperate in response to DNA damage. It can be proposed that PARP and DNA-PK co-localize onto the same DNA lesion, resulting in the stimulation of their respective ribosylation and kinase activities. PARP may then ADP-ribosylate DNA-PKcs to further stimulate DNA-PK kinase activity during the process of responding to DNA damage (Ruscetti *et al.*, 1998).

Additional evidence that DNA-PK and PARP are critical molecules in DNA repair comes from studies on programmed cell death or apoptosis. Cells undergoing this process display distinctive biochemical characteristics including fragmentation of chromatin that appears as a DNA ladder when chromatin DNA is fractionated on agarose gels (reviewed in White, 1996). Apoptosis occurs during development of the immune system, or can be induced by treatment of cells with chemotherapeutic agents such as ionizing radiation and cisplatin. Both PARP and DNA-PKcs are proteolytically cleaved by the cysteine protease called CPP32 (or Caspase 3) early in apoptosis, at about the same time as inter-nucleosomal DNA breakages are detected. PARP is cleaved to form 89 and 24 kDa polypeptides (Lazebnik *et al.*, 1994; Nicholson *et al.*, 1994), while DNA-PKcs is cleaved to produce a N-terminal fragment of 240 kDa and a C-terminal fragment of 150 kDa. The 150 kDa fragment is further cleaved to yield two smaller polypeptides (120 kDa and 30 kDa) (Song *et al.*, 1996; Casciola-Rosen *et al.*, 1996). Cleavage renders both enzymes inactive, while Ku remains unaffected throughout this process. Inactivation of potential DNA damage sensors such as DNA-PKcs and PARP may remove the cell's ability to recruit the DNA repair machinery to allow for rapid degradation of chromatin.

It is unlikely that PARP is involved in any checkpoint pathways because PARP knockout mice show no obvious cell cycle control defects in response to DNA damage (Wang *et al.*, 1995). Although very preliminary, the most attractive speculation for PARP function in DNA dsb repair would be that the production of ADP ribose polymers is required to preclude homologous recombination pathways in order to allow NHEJ and DNA-PK function (Morrison *et al.*, 1997).

c) Synopsis of the role for DNA-PK in DNA damage response pathway

It is certain that DNA-PK is required during the process of the NHEJ pathway in

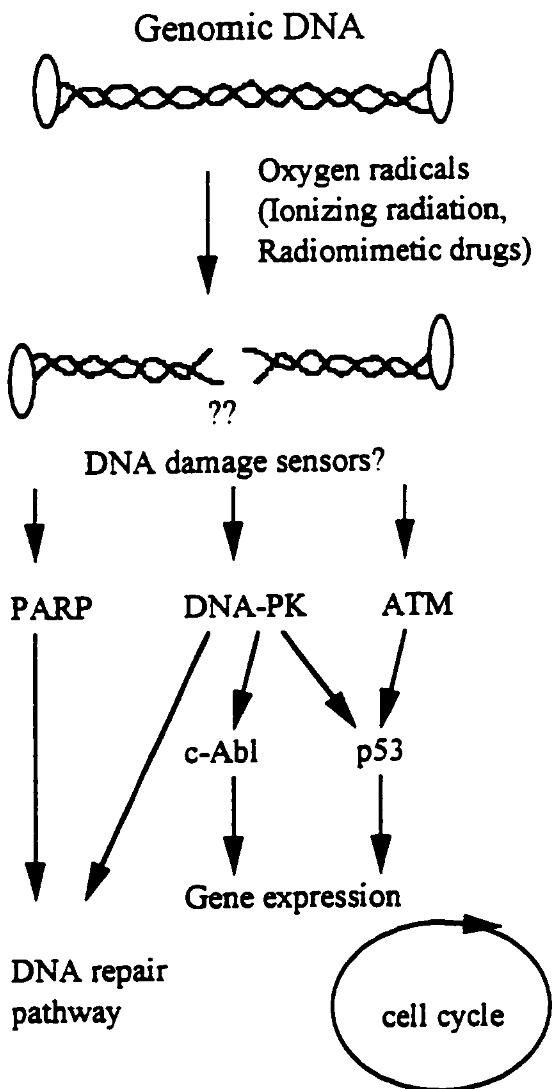

Figure I-5: Role of DNA-PK in DNA damage response. The presence of oxygen radicals induces genomic dsDNA breaks. DNA-PK and ATM become activated either directly by dsDNA breaks or indirectly through a DNA damage sensor protein; in turn, DNA-PK and ATM activate p53 and/or c-Abl to regulate expression of genes involved in DNA repair and/or cell cycle control. In response to DNA damage, PARP becomes activated, and possibly in cooperation with DNA-PK, activates a parallel DNA-repair pathway.

DNA dsb repair. However, the role of DNA-PK in DNA damage sensing and signaling pathways remains unclear (Figure I-5). It is likely that along with ATM and possibly other kinases, DNA-PK acts upstream of p53 and targets p53 and/or c-Abl in response to DNA damage. Whether or not DNA-PK and ATM are immediate sensors of DNA dsbs is uncertain. It is very likely that both kinases act in parallel pathways that cooperate and impinge on each other to ensure a proper cell cycle checkpoint response. PARP likely acts in another parallel pathway, possibly in cooperation with DNA-PK, that signal directly to or recruit DNA repair proteins. Alternatively, DNA-PK may induce expression of certain DNA repair genes via regulating c-Abl activity.

I.11: DNA-PK and the control of transcription

In addition to its role in NHEJ and DNA damage response pathways, several studies support a role for DNA-PK in transcription. DNA-PKcs and Ku have been shown to exist in a complex with the RNA polymerase II holoenzyme that includes basal transcription factors and DNA repair proteins (Maldonado *et al.*, 1996). DNA-PK also phosphorylates the TATA-box binding protein (TBP) of TFIID and the CTD of RNA polymerase II *in vitro* (Dvir *et al.*, 1992, 1993; Labhart, 1996). Further, the presence of DNA-PK represses transcription of ribosomal genes by RNA polymerase I in *Xenopus* (Labhart, 1995) and human cells (Kuhn *et al.*, 1995). Although the exact biochemical function of DNA-PK in these transcription systems is unknown, it is attractive to postulate that DNA-PK may regulate transcription through phosphorylation of TBP and/or the CTD of RNA polymerase II (Labhart, 1996).

DNA-PK activity is stimulated by transcriptional activators, including the GAL4 DNA-binding domain and the human heat shock factor, HSF1 (Peterson *et al.*, 1995a). It was recently demonstrated that HSF1 interacts with Ku and DNA-PKcs in an ELISA based protein-protein interaction assay (Huang *et al.*, 1997). The consequence of this interaction was a 5 fold increase of DNA-PK activity towards the CTD of RNA polymerase II. HSF1 was also able to stimulate DNA-PKcs activity in the absence of Ku (Huang *et al.*, 1997). Further, overexpression of Ku70 in rat cells suppresses expression of the 70 kDa heat shock protein, hsp70 (Yang *et al.*, 1996). It has been proposed that the Ku protein binds to heat shock elements (HSE) and displaces HSF1. As a result, hsp70

gene expression is repressed (Li *et al.*, 1995; Kim *et al.*, 1995). Taken together, these data suggest a role for DNA-PK in the heat shock response, which may be relevant to a phenomena known as thermal radiosensitization. It has been long known that heat treatment of cells increases their sensitivity to ionizing radiation. This phenomena could be attributed to the competition between the heat shock response and DNA repair pathways for DNA-PK. Lending support to this theory, it has been observed that the radiosensitivity of *scid* cells does not increase following heat treatment (reviewed in McConnell and Dynan, 1996).

As described earlier, DNA-PK phosphorylates the glucocorticoid receptor (GR) and the octamer transcription factor-1 (Oct-1) only when the proteins are in contact with the negative regulatory element (NRE1) of the mouse mammary tumor virus (MMTV) promoter (Giffin *et al.*, 1996). Phosphorylation of GR by DNA-PK resulted in the repression of transcription from the MMTV promoter directed by RNA polymerase II. Efficient phosphorylation of GR by DNA-PK occurs only when both are co-localized to the NRE1 element (Giffin *et al.*, 1996; 1997). These data represent the most convincing data for DNA sequence specific stimulation of DNA-PK activity.

I.12: DNA-PK and chromatin structure

DNA within the nucleus is wrapped around histone proteins to form a tightly packed DNA-protein complex known as chromatin. The role of the chromatin structure in the regulation of transcription in higher eukaryotes has become abundantly clear in recent years (reviewed in Gregory and Hörz, 1998). It is very likely that the study of DNA repair and V(D)J recombination in the future will involve investigations on how these processes are carried out in the physiological context of the chromatin. A role for DNA-PK in modulating chromatin structure is suggested by the involvement of Ku in telomere maintenance in yeast and the identification of a histone acetyltransferase protein that associates with DNA-PK.

Telomeres are structures that contain distinctive short repeats of DNA sequences and are found at the ends of linear eukaryotic chromosomes. Their function is to prevent the loss of DNA from the ends of chromosomes, since DNA polymerase cannot completely replicate a linear DNA molecule. Telomeres may also prevent unwanted

recombination with other chromosomal termini. Following each DNA replicative cycle, the enzyme telomerase adds DNA sequences to the telomeric ends, via an RNA-templated reaction, in order to prevent their erosion. Shortening of telomeric ends has been associated with chromosome instability and cellular senescence (reviewed in Johnson *et al.*, 1998).

In budding yeast, telomeres consist of tandem arrays of the consensus sequence $C_{(1-3)}A$. Genes that are required for telomere maintenance include the genes encoding the telomerase components *TLC1*, *EST1*, *EST2*, *EST3* and *EST4/CDC13* and a $C_{(1-3)}A$ sequence specific binding protein call Rap1p (reviewed in Zakian, 1996; Weaver, 1998). In addition to functioning in the maintenance of chromosome integrity, yeast telomeres will repress transcription of genes placed adjacent or within them. This phenomenon, known as the telomere position effect (TPE), has been proposed to be involved in the transcription control of certain genes, such as the genes of the mating switch loci in yeast (Shore, 1995). The best characterized genes implicated in TPE are *SIR2*, *SIR3*, and *SIR4* (Aparicio *et al.*, 1991). Since the Sir proteins do not contain DNA binding motifs, it is thought that they are targeted to the telomeres by an interaction with Rap1p. Once positioned at telomeres, the Sir proteins and Rap1p repress transcription by, in part, packaging DNA into a heterochromatin like state (Wiley and Zakian,1995).

It was originally noted that *yku80* or *yku70* mutants exhibited shortening of the telomeres (Boulton and Jackson, 1996a,b). Recently, using a genetic screen to identify additional proteins that control telomerase activity, two groups have reported that mutations to *yku80* or *yku70* in combination with *cdc13* or *est1* is a lethal event (Nugent *et al.*, 1998; Polotnianka *et al.*, 1998). Furthermore, the yeast genes *MRE11* and *RAD50* were found to be epistatic to two telomerase subunit genes, *EST2* and *CDC13*, whereas the *YKU80/70* genes were not (Nugent *et al.*, 1998). This implies that *MRE11-RAD50-XRS2* act in the same pathway as telomerase, whereas Ku may act in a parallel pathway. Insights to the nature of this pathway were provided by Gasser and colleagues (Laroche *et al.*, 1998). They reported that mutations to *YKU70* or *YKU80* disrupt the normal punctate staining of telomeric DNA and the telomere associated proteins Rap1p, Sir3p and Sir4p at the nuclear periphery. In the absence of Ku, the immuno-staining for Sir3p, Sir4p and Rap1p disperse throughout the nucleoplasm, as if the normal telomeric

clustering at the nuclear periphery has been lost. Sir4p has previously been shown to interact with Ku by yeast two hybrid assays, suggesting that interaction of Ku with the Sir proteins and Rap1p is important for their functions (Tsukamoto *et al.*, 1997). Consistent with this idea, TPE is lost only in *yku80* or *yku70* mutant strains, but not *mre11* or *rad50* mutant strains (Boulton and Jackson, 1998; Laroche *et al.*, 1998).

Taken together, these data show that Ku is crucial in maintaining the integrity of the telomere and chromosome ends in yeast. It seems that Ku participates with Rap1p, Sir2p, Sir3p and Sir4p in telomeric repression and cooperates with Mre11/Rad50/Xrs2 to provide a suitable substrate for telomerase activity. The Mre11/Rad50/Xrs2 complex may be involved in nucleolytic processing of the $C_{(1-3)}A$ repeats, much like its proposed 3' to 5' resecting activity in NHEJ. As alluded to earlier, mutations to *SIR2, 3* and *4* have been shown to sensitize *rad52* cells to ionizing radiation (Tsukamoto *et al.*, 1997); however, their roles in NHEJ remain to be determined. It is possible that these proteins form a heterochromatin-like state at the ends of DNA during NHEJ, as seen during TPE (Weaver, 1998). Interestingly, cells containing mutations to *TEL1*, a yeast gene similar to DNA-PKcs, also display shortened telomeres. However, *TEL1* is not required for NHEJ and has little effect on TPE (Boulton and Jackson, 1998). These studies suggest that Ku and possibly DNA-PKcs may have a role in maintaining the integrity of telomeres in higher eukaryotes.

Yeast two hybrid assays and antibody co-immunoprecipitation studies have shown that human Ku70 associates with the human homologue of the yeast GCN5 histone acetyltransferase (HAT) (Barlev *et al.*, 1998). Histone acetyltransferases catalyze the transfer of an acetate moiety from acetyl-CoA onto the ε-amino group of lysine residues in the N-terminal tail of histones. The acetylation of histones is believed to alter histone-DNA or histone-histone interactions, thereby remodeling the chromatin (reviewed in Kuo and Allis, 1998). DNA-PK is able to phosphorylate hGCN5 and repress its HAT activity *in vitro* (Barlev *et al.*, 1998). Hence, one way in which DNA-PK may be involved in regulating chromatin structure is by regulating HAT activity (Barlev *et al.*, 1998).

I.13: Aims of Thesis

Although much is known about the genetics and biochemical properties of DNA-PK, its precise function in DNA dsb repair, V(D)J recombination and the regulation of transcription remains elusive. One can postulate several general models as to how DNA-PK may act in these processes. First, once DNA-PKcs is targeted to DNA by Ku, DNA-PK may act as a signaling molecule via phosphorylation of other molecules. Second, since DNA-PKcs is such a large polypeptide, it may act as a scaffolding protein to recruit other proteins and subsequently mediate their activity. Third, DNA-PKcs and Ku may be involved in establishing a heterochromatin structure suitable for DNA repair by NHEJ, while precluding other events such as transcription or recombination. In doing so, DNA-PK may also play a structural role in modulating DNA conformation to allow for suitable DNA processing events.

It is therefore important to understand how DNA-PK interacts with DNA and with other proteins. To this end we sought to identify novel proteins that interact with DNA-PK. During the initial purification of DNA-PK from Hela cells, several polypeptides that were phosphorylated in a DNA dependent manner were present in a partially purified active kinase protein fraction that contained DNA-PKcs (Lees-Miller *et al.*, 1990). One of these polypeptides was identified as Ku, which is now known to be the DNA-targeting component of DNA-PK. The other polypeptides were therefore considered to be plausible candidates for proteins that might also interact with DNA-PKcs and possibly regulate its activity. The focus of this thesis was three fold:

1) To purify and identify the substrates that were present in the partially purified preparation of DNA-PK.
2) To study the possible interactions between the identified substrates with DNA-PKcs, Ku and DNA.
3) To study the effects of this interaction on DNA-PK activity and on any known functions of the identified substrates.

CHAPTER II

MATERIALS AND METHODS

Chapter II - Materials and Methods

II.1: Protein purification

All proteins were purified from human placenta as illustrated in Figure II-1 and as described in Chan *et al.*, (1996) and Ting *et al.*, (1998). All procedures were performed at 4°C up until steps involving FPLC column chromatography. The composition and names of the buffers used are listed in Table II-1. All buffers were supplemented with 0.2 mM PMSF and 1 mM DTT, except for the 4 x SDS sample buffer, and unless otherwise indicated. Buffers used before the first anion exchange chromatography step (DEAE) also contained protease inhibitors leupeptin and aprotinin at 1 µg/mL each. Purification of proteins during the procedure was monitored by SDS-PAGE and Western blot analysis.

Human placentas, from normal births or from cesarean operations, were obtained from the Maternity Ward at Foothills Hospital, Calgary. Use of human placenta was carried out with permission from and in accordance with, the Biohazards, BioSafety and Bioethics committees of the University of Calgary, and the Medical Research Council of Canada. The placenta was transported back to the laboratory in an ice-filled container and immediately washed with ice cold PBS and cut into small 2 cm^3 pieces. The cubes were frozen using liquid nitrogen, weighed and distributed into 250 g portions. These portions were stored at -80°C for no longer than 2 weeks before further use. The average size of a placenta was about 500 g.

250 g of frozen 2 cm^3 pieces of human placenta were partially thawed at 4°C for 2 to 4 hours and then homogenized using a PRO250 Handheld Homogenizer (PRO Scientific, Inc./ Bio-Rad) at 5 x 30 sec. bursts in homogenization buffer (200 mL per 100 g of placenta tissue). The homogenized extract was centrifuged at 10,000 x g for 20 min. The supernatant was removed, and the pellet was subjected to two more rounds of homogenization using the same buffer. Each time, the rehomogenized extract was pelleted by centrifugation, and all the supernatants were combined. Solid ammonium sulfate was gradually added to the combined placenta extract to 20% saturation and allowed to stir for 1 hour at 4°C. The extract was then centrifuged at 10,000 x g for 30 min. and the supernatant recovered. Ammonium sulfate was added to the supernatant to 60% saturation and allowed to dissolve by stirring for another hour as before. The

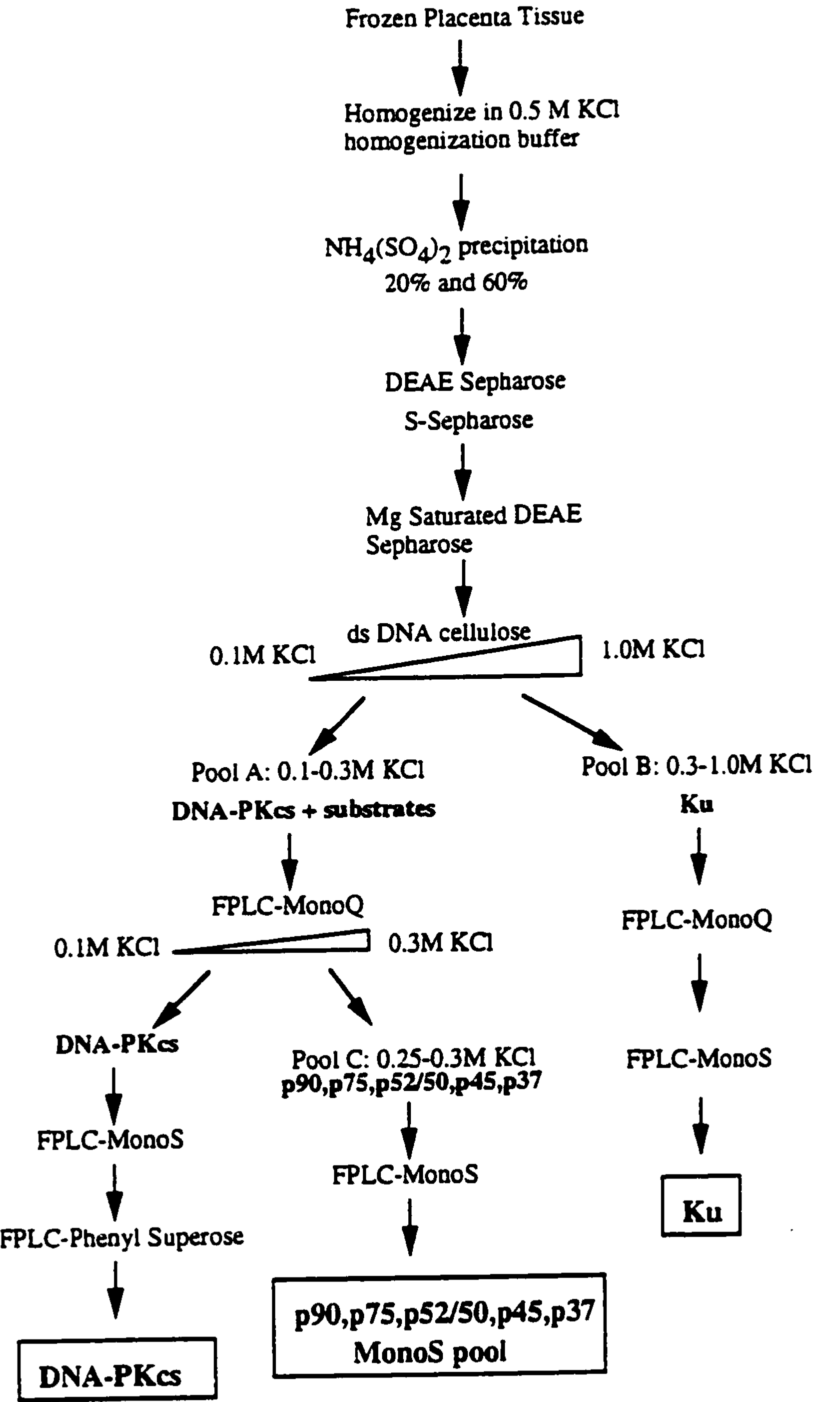

Figure II-1: Scheme for the purification of DNA-PKcs, Ku70/80 and associated substrates from human placenta. The protocol used were as described in Chapter II (Materials and Methods). Before each chromatography step, samples were dialyzed into the buffer used to pre-equilibrate the column. Columns where a linear gradient of salt concentration was used to elute bound proteins are represented by triangles. Purified proteins, highlighted by a box, were concentrated using microconcentrators and stored in aliquots at -80°C.

Table II-1: Contents of buffers used for the purification of proteins from human placenta.

Buffers	Composition
PBS	136 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2P0_4, pH 7.2
Homogenization buffer	500 mM KCl, 50 mM Tris, 20 mM $MgCl_2$, 10% glycerol (v/v), 2 mM EDTA, pH 7.9
TB	50 mM Tris-HCl, 5% glycerol (v/v), 0.2 mM EDTA, pH 8.0
PS-A	1 M ammonium sulfate, 50 mM KCl, 50 mM Tris-HCl, pH 8.0
PS-B	50 mM KCl, 50 mM Tris-HCl, pH 8.0
HPB	25 mM Hepes, 5% glycerol (v/v), 0.2 mM EDTA, pH 7.5
4 x SDS sample buffer	3.2% SDS, 10 mM DTT, 40% glycerol, 320 mM Tris-HCl, pH 6.8

ammonium sulfate saturated extract was again centrifuged at 10,000 x g for 30 min., and this time the pellet was retained. The pellet was gently resuspended in a minimal volume of TB buffer and dialyzed into TB buffer containing a salt concentration of 50-100 mM KCl. The dialyzed placental extract was then loaded onto a 6 x 30 cm column of DEAE-fast flow resin (Pharmacia) that had been pre-equilibrated in 100 mM KCl TB buffer. The column was washed with the same buffer and eluted stepwise with 1.0 M KCl TB buffer. The step-eluted sample was dialyzed to a salt concentration of 50-100 mM KCl in TB as described before. The dialyzed DEAE step-eluted sample was then applied to a 2.5 x 15 cm column filled with S-Sepharose fast flow resin that had been equilibrated in 100 mM KCl TB buffer and processed as before. The S-Sepharose 1.0 M KCl eluent was dialyzed against 100 mM KCl TB buffer containing 20 mM $MgCl_2$ and subjected to a second round of chromatography on DEAE resin using a column (2.5 x 3.5 cm) that had been pre-equilibrated with the same buffer. Flow through fractions which contained DNA-PKcs, Ku and the DNA-PK substrates, were pooled and briefly dialyzed (30 min.) against 100 mM KCl TB buffer to remove the $MgCl_2$. At this time, the Mg-DEAE flow-through sample was divided into three separate pools for further purification.

Each of the dialyzed "Mg-DEAE flow-through" pools was separately applied to a 2 x 4 cm column of dsDNA-cellulose resin that had been pre-equilibrated with 100 mM KCl TB. The column was washed in the same buffer and proteins were eluted with a 30 mL linear gradient of 0.1 M to 1.0 M KCl TB buffer. Under these conditions, the majority of DNA-PKcs eluted between a salt concentration of 100 mM to 300 mM KCl, while Ku eluted at salt concentrations greater than 300 mM. Fractions containing DNA-PKcs and Ku were pooled separately and referred to as pools A and B, respectively. In addition to DNA-PKcs, pool A contained six other prominent polypeptides that were easily seen in Coomassie Blue stained SDS-PAGE gels. They included polypeptides that migrated with molecular masses of approximately 90-, 75-, 52-, 50-, 45- and 37-kDa. Pool A and B from each of the three separate dsDNA-cellulose chromatography steps were combined and further purified using FPLC-chromatography.

Polypeptides in pool A were dialyzed accordingly and further fractionated on a MonoQ FPLC HR5/5 column (Pharmacia) in TB buffer containing 0.02% Tween (v/v). A linear gradient of 100 mM to 500 mM at 4 mM KCl/min. was used to elute bound

proteins. Fractions eluting between 150 to 200 mM KCl containing DNA-PKcs were pooled, dialyzed, and applied to a MonoS-FPLC HR5/5 column (Pharmacia) using the same protocol as described for the MonoQ-FPLC step.

The hydrophobic interaction chromatography column, Phenyl Superose-FPLC HR5/5 (Pharmacia) was used as a final step in order to obtain a completely homogeneous preparation of DNA-PKcs. Fractions containing DNA-PKcs from the MonoS-FPLC column were pooled and dialyzed against PS-A buffer and applied onto a Phenyl Superose-FPLC column that had been pre-equilibrated with the same buffer. Bound polypeptides were eluted using a linear gradient of PS-A to PS-B, at 0.7% PS-B/min. DNA-PKcs eluted in fractions between 650 mM to 750 mM ammonium sulfate. These fractions were pooled and dialyzed against HPB buffer containing 100 mM KCl. The dialyzed fractions were concentrated using Centricon 100 microconcentrators (Amicon) and stored in aliquots at -80°C.

The six other predominant polypeptides in Pool A separated from DNA-PKcs on the MonoQ-FPLC column at salt concentration of 250 mM to 300 mM KCl. These fractions were combined to form pool C, dialyzed and further processed using MonoS-FPLC employing the same conditions as described for the MonoQ-FPLC step. The 90-, 75-, 52-, 50-, 45- and 37 kDa polypeptides eluted as a broad peak (200 mM to 300 mM KCl) from the MonoS-FPLC column. Fractions containing these polypeptides either were used separately or combined to form the MonoS pool as indicated. Combined fractions (MonoS pool) were dialyzed into HPB containing 100 mM KCl, concentrated using Centricon 30 microconcentrator (Amicon), and stored as aliquots at -80°C.

Ku was purified to homogeneity from pool B using MonoQ-FPLC and MonoS-FPLC. Briefly, pool B from dsDNA-cellulose chromatography was dialyzed against 100 mM KCl TB and chromatographed on MonoQ-FPLC as previously described for pool A. Fractions containing Ku were combined, dialyzed and passed over MonoS-FPLC using chromatographic conditions described for purifying DNA-PKcs and pool C. However, unlike the polypeptides in pool C and DNA-PKcs, Ku did not bind the MonoS-FPLC column, and flow through fractions containing Ku were immediately combined and concentrated using Centricon 100 microconcentrators (Amicon). Homogeneous preparations of Ku (as judged by SDS-PAGE and Western blot) were stored in aliquots

at -80°C.

Purified protein concentrations were determined using a protein dye assay (Bio-Rad), using bovine serum albumin (BSA) as a standard according to the manufacturer's guide. Purified rat liver eIF-2 and recombinant eIF-2α were a kind gift from Dr. Scott Kimball, Pennsylvania State University. Purified total core histones and mononucleosomes from Hela cells were a kind gift from Dr. C. David Allis, University of Rochester.

II.2: DNA-PK assay and phosphorylation reactions

Phosphorylation reactions were carried out as described in Lees-Miller *et al.*, (1990) and Chan *et al.*, (1996). 1-2 μg of substrate protein was incubated with or without purified DNA-PKcs (0.3 μg) and Ku (0.1 μg) in the presence or absence of 10 μg/mL sonicated calf thymus DNA in a final reaction volume of 20 μL. Reactions contained the following: 25 mM Hepes (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT and 0.25 mM ATP ($[^{32}P]\gamma$-ATP, specific activity 500-1,000 d.p.m./pmol {Easy-tide, NEN Life Science Products}). The reactions were started by the addition of ATP, incubated at 30°C for 10 min., and stopped with 10 μL of 4 x SDS sample buffer (contents described in Table II-1). The mixtures were boiled, fractionated on SDS-PAGE (10% acrylamide:0.2% bisacrylamide), stained with Coomassie Blue, dried and exposed to X-ray film (Fuji) at RT overnight. Phosphorylated polypeptides were viewed following development of X-ray film.

Assays for DNA-PK activity were performed under the same conditions except 250 μM of the synthetic peptide PESQEAFADLWKK was added to the reaction mixture in place of the protein substrate. Reactions were stopped by addition of an equal volume of 30% Acetic Acid (v/v)/5 mM ATP. The reaction mixture was then spotted onto 2 cm^2 squares of p81 phosphocellulose filter paper (Whatman) in duplicate (15 μL each). Filters were washed four times with approximately 500 mL of 15% acetic acid. Incorporated radioactivity (^{32}P) bound to the filters were measured by Cerenkov radiation using a scintillation counter (Beckman LS6500) set at wide open window.

II.3: Electrophoresis and immunodetection

The gel recipe/conditions for SDS-PAGE were as described in Lees-Miller and Anderson (1989). The conditions used for electrophoresis and Western blots were dictated by the molecular mass of the polypeptide of interest. Polypeptides greater than 150 kDa, for example DNA-PKcs, were fractionated on 7.9% acrylamide:0.1% bisacrylamide SDS gels, and transferred onto PVDF (polyvinylidenediflouride) membrane (Micron separations, Inc.) using transfer buffer containing 25 mM Tris base, 185 mM glycine, 20% methanol and 0.05% SDS (w/w) at 100 V for 1 hour at RT. Polypeptides smaller than 150 kDa were separated on 10% acrylamide:0.2% bisacrylamide SDS gels and transferred to PVDF membrane with buffer containing 25 mM Tris base, 200 mM glycine, 20% methanol for 35 min. at RT. Transfer buffers were pre-chilled at 4°C before use. Following transfer, the PVDF membranes were blocked for at least one hour or overnight at RT in 5% (w/v) skim milk (Non-Fat, Lucerne) in TTBS (20 mM Tris-HCl, pH 7.5, 500 mM KCl, 0.5% Tween 20 (v/v)). The blots were then washed three times for 10 min. in TTBS. The conditions employed for the primary antibodies are summarized in Table II-2. Following primary antibody treatment, the blots were again washed as before and incubated with the required secondary antibody at a dilution of 1:3000 in TTBS for 30 min. at RT. The blots were then extensively washed five times for 10 min. each wash, using TTBS, before being processed using Enhanced Chemiluminescence (ECL, Amersham) according to the manufacturer's guide.

II.4: Transfer of proteins for N-terminal amino acid sequence analysis

The MonoS pool of proteins containing approximately 20 μg of each polypeptide were initially fractionated on 10% acrylamide:0.2% bisacrylamide SDS-PAGE that had been pre-aged for 24 hours at RT. Polypeptides were then transferred onto PVDF membrane (Bio-Rad Sequencing grade, 0.2 μm) in 10 mM CAPS, 10% methanol (v/v), pH 11 at 100 V for 75 min. at RT as described by Matsudaira (1987). The PVDF membrane was stained briefly with freshly prepared Coomassie Blue and destained in 50% (v/v) methanol / 0.05% (v/v) acetic acid. The membrane was then washed with water and allow to air dry. The 52/50- and 37 kDa polypeptide bands were excised and sent to University of Wisconsin Biotechnology Center (Madison, Wisconsin), while the

Table II-2: Names and conditions for antibodies used in this study.
All antibodies were stored at -20°C and diluted with TTBS prior to use.

Polypeptide	**Antiserum**	**Dilution of antibody and time of Incubation at RT**
DNA-PKcs	Rabbit DPK1 (Anderson and Lees-Miller, 1992) Monoclonal 18-2 and 42-27 (a gift from Dr. Tom Shenk, Princeton University.)	1:3,000 for 1 hr. 1:1,000 for 1 hr.
Ku	Mouse polyclonal HKu (Chan & Lees-Miller, 1996)	1:2,500 for 1 hr.
NF90/45	Rabbit polyclonal NF90/45 (Kao *et al*, 1994) Mouse polyclonal NF90/45 (R.Ye, this study)	1:3,000 for 1 hr. 1:3,000 for 1 hr.
eIF-2β, 2γ	Rabbit polyclonal 2β, 2γ (a gift from Dr. John W. Hershey, UC Davis)	1:10,000 for 1 hr.
human GCN5	Rabbit polyclonal hGCN5 (a gift from Dr. C. David Allis, University of Rochester)	1:2,000 for 1 hr.

75- and 45 kDa polypeptide bands were sent to University of Victoria Sequencing Center (Victoria, British Columbia) for protein sequence analysis.

II.5: Plasmid and oligonucleotide probe preparation

The closed-circular plasmid vector pGEM 7Zf+ (Promega) was propagated in *E.Coli* strain JM109, harvested and purified using Qiagen-tip columns (Qiagen) according to the supplier's guide. Linearized plasmids were obtained by digestion of the pGEM plasmid with *Bam*H1 (New England Biolabs) according to manufacturer's instructions. Restriction enzyme was inactivated by heating to 60°C for 5 min. after digestion was complete.

A 40 bp deoxyoligonucleotide with the sequence 5'-CCCAATTCGCCCTATAG-TGAGTCGTATTACAATTCACTGG-3' was synthesized and gel purified by the DNA Sequence Facility, University of Calgary. The 40 bp oligonucleotide sequence corresponded to a 40 bp product of *Hae*III digested pGEM plasmid and contained no known specific DNA binding sequences. To produce the blunt ended 40 bp DNA duplex, equimolar amounts of the upper and lower strand oligonucleotides were resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), combined at equal molar ratios, and heated to 97°C for 10 min. The sample was then allowed to cool overnight at RT.

The 40 bp DNA duplex was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Life Technologies, Inc.) according to the manufacturer's instructions. The reaction mixture was applied to Microspin-G50 columns (Pharmacia) to partition the labeled 40 bp probe and unincorporated nucleotides as described in the supplier's guide. The amount of radioactive phosphate incorporated into the probe was counted using a scintillation counter. The labeled probe was stored in TE at -20°C for up to two weeks and was diluted with TE to approximately 15,000-20,000 d.p.m. per μL prior to use.

In gel shift competition reactions, the competitor DNA was added together with radiolabeled DNA probe, unless otherwise indicated. The fold excess of competitor DNA over the DNA probe was calculated assuming 100% recovery of DNA probe after removal of unincorporated nucleotide.

II.6: Electrophoretic mobility shift assay

The protocol used for the electrophoretic mobility shift assay (EMSA) was modified from the procedure used by Blier *et al.* (1993). DNA binding reactions were performed by incubating the ^{32}P-labeled 40 bp DNA (approximately 0.3 ng DNA [15 fmol], 40,000 d.p.m.) with purified proteins for 10 min., in a final reaction volume of 20 μL containing 25 mM Hepes pH 7.5, 50 mM KCl, 10% glycerol, 2 mM DTT. Samples were next analyzed by fractionation on non-denaturing gels (3.9% acrylamide:0.1% bisacrylamide) in 50 mM Tris, 0.382 M glycine buffer. The non-denaturing gels were prerun for 1 hour at 200 V prior to sample loading and samples were subjected to electrophoresis for 2 hours at the same voltage at RT. Gels were then dried and processed by autoradiography.

Where indicated, chemical cross-linkers were added to the samples after the initial 10 min., and incubated for a further 10 min., prior to electrophoresis. Two types of chemical cross-linkers were used: Bis(sulfosuccinimidyl) suberate [BS^3] (Pierce Chemicals), which is a non-cleavable, water soluble thiol cross-linker of spacer length 11.4Å; and Glutaraldehyde (E.Merck, 25%, electron microscopy grade) which is a non-specific protein-protein cross-linker. BS^3 was prepared in either DMSO or water prior to use and added to the binding reaction to a final concentration of 1.0 mM. Glutaraldehyde was diluted in water to 2.5% (v/v) prior to use and added to the binding reactions to a obtain a final concentration of 0.06% (v/v).

Typical protein concentrations used for each EMSA reaction are as follows, unless otherwise indicated: Ku, 5-10 ng; DNA-PKcs, 15-30 ng; Mono S-FPLC column fractions, 50 ng; MonoS pooled fractions, 30 ng. For EMSA studies using crude cell extracts, typically 1 or 2 μg of total proteins were used for each reaction. For EMSA studies using antibodies, approximately 2-4 μg of purified Ig proteins were added together with purified proteins before addition of the probe.

II.7: Purification of antibodies

Total IgG was purified from rabbit pre-immune or DPK1 anti-sera using methods described by Harlow and Lane (1988). Briefly, the pH of the crude serum was adjusted to pH 8.0 by adding one-tenth the volume of 1.0 M Tris-HCl, pH 8.0. The serum was

then applied onto a 1 cm x 1 cm protein A-Sepharose (Pharmacia-Biotech) column that had been pre-equilibrated with 100 mM Tris-HCl, pH 8.0. The column was washed consecutively using 10 mL of 100 mM Tris-HCl, pH8.0, followed by 10 mL of 10 mM Tris-HCl, pH 8.0. Bound IgG proteins were eluted with 100 mM glycine pH 3.0, and the pH of the samples was immediately adjusted to 7.0 by adding one-tenth the volume of 1.0 M Tris-HCl, pH 8.0. Purified IgG proteins were quantitated using BioRad protein assay and stored at 4°C for up to a month.

II.8: Preparation of recombinant protein expression constructs

Expression of recombinant NF90 and NF45 (rNF90/rNF45) was performed using the pQE expression system (Qiagen) according to Kao *et al.*,(1994). The pQE vectors have a multiple cloning cassette that allows addition of six histidine residues onto the C-terminus of the recombinant protein. *E. coli* strain, M15, which contained a repressor plasmid pREP4, was used to propagate the pQE vectors. Unique restriction enzyme (RE) sites within the NF90 cDNA sequence were utilized to construct the rNF90 deletion polypeptides. The pQE-90 expression vectors constructed by Ruiqiong Ye, a technician in our laboratory, are described below and are summarized in Table II-3.

For the pQE-90FL (wild type, full length) construct, the 2013 bp full length NF90 cDNA flanked by *Bgl*II and *Hind*III restriction enzyme sequences was ligated into pQE9 that had been digested with *Bam*HI and *Hind*III. Expression of the pQE-90FL yielded a protein product of 1-617 amino acids in length. To generate the pQE-90D construct, a 240 bp *Hind*III fragment was removed from pQE-90FL construct, and the pQE9 plasmid was religated. The protein product expressed from pQE-90D was called rNF90D and included amino acids 1-591. The pQE-90RV construct was derived from digestion of the pQE-90D plasmid with *Hind*III followed by a Klenow polymerase reaction to fill in the 3' recessed termini. The plasmid was subjected to a second digestion with *Eco*RV to excise out a 831 bp *Eco*RV-*Hind*III fragment. The religated plasmid yielded the expression construct pQE-90RV which coded for the protein rNF90RV with amino acids 1-314. pQE-90H3 was constructed from a 831 bp *Eco*RV-*Hind*III fragment from pQE-90D which was inserted into pQE32 vector digested with *Bam*HI and *Hind*III and filled in with Klenow fragment to create the necessary blunt ends for ligation. Expression of

Table II-3: Recombinant NF90 deletion fragments. Different restriction enzymes were used to create pQE expression constructs of rNF90 containing different deletions. Listed below are the names of the rNF90 polypeptides containing the deletions, the number of amino acids in each of the polypeptides and the restriction enzyme used to generate the expression constructs.

Name of rNF90 deletion fragment	**Restriction Enzymes used**	**Amino Acids**
rNF90-FL	N/A	1-671
rNF90-D	*Hind*III	1-591
rNF90-RV	*Eco*RV	1-314
rNF90-H3	*Eco*RV and *Hind*III	314-591
rNF90-PH	*PstI* and *Hind*III	417-591
rNF90-SP	*Sal*I and *Pst*I	152-417

pQE-90H3 yielded rNF90H3 with amino acids 314-519.

To construct pQE-90SP and pQE-90PH, pQE-90SH was initially created by digesting pQE-90FL with *SalI* and *Hind*III to create a 1323 bp fragment that was religated back into pQE9 that had been digested with the *Bam*HI and *Hind*III. The pQE-90SP construct was derived from a 798 bp *SalI*-PstI fragment from pQE-90SH inserted into pQE31 that had been digested with *SalI* and *PstI*. The protein product of pQE-90SP was rNF90SP which included amino acids 152-417. Finally, pQE-PH was a product of the insertion of a 525 bp *PstI*-*Hind*III fragment from pQE-90SH into the pQE31 vector that had been digested with both *PstI* and *Hind*III. Expression of pQE-90PH yielded a protein with amino acids 417-519, termed rNF90PH. These protein constructs are shown diagramatically in Chapter V, Figure V-5.

These constructs were transformed into competent M15 bacterial cells and selected using LB (Luria Broth) plates containing 75 μg/mL of Ampicillin and 25 μg/mL of Kanamycin. Positive colonies were selected and grown in LB media containing the same antibiotics.

II.9: Purification of recombinant proteins

5 mL of M15 bacterial culture harboring pQE expression vectors for full length NF45, NF90 or NF90 restriction polypeptides were grown overnight at 30°C in a shaker incubator (Eviron-Shaker, Lab-Line). The culture was then inoculated into 250 mL LB media and incubated at 37°C in the shaker for at least 5 hours, or until the optical density reading at A_{660} was at least 0.6. Expression was induced by allowing the bacteria to grow for a further 2 hours after the addition of IPTG (0.5 mM). The cells were harvested and pelleted by centrifugation for 10 min., at 10,000 x g at 4°C. The pellet was resuspended in 50 mM KCl HPB buffer and sonicated at 6 x 45 sec. pulses, on ice, using a micro ultrasonic cell disrupter (Kontes) at 60 MHz. Sonicated cells were centrifuged for 10 min. at 10,000 x g at 4°C and the supernatant discarded.

rNF45, rNF90 and the rNF90 deletion polypeptides were present in the pellet as inclusion bodies and were therefore insoluble. These proteins were resolubilized in 8 M urea in 50 mM Tris-HCl, pH 8.0, and diluted with an equal volume of 50 mM Tris-HCl, pH 8.0 to obtain a final urea concentration of 4 M. The recombinant proteins were then

applied to a chelating column (Ni^{2+}-NTA resin, Qiagen) that had been pre-equilibrated using the same 4 M urea buffer. After loading, the column was washed with 4 M urea, 50 mM Tris-HCl pH 8.0 and then with 10 mM immidazole in water. Bound recombinant proteins were eluted using 200 mM immidazole and immediately dialyzed into TB buffer containing 100 mM KCl at 4°C. Purity was estimated at >95% by Coomassie Blue staining. Purified proteins were stored in aliquots at -80°C.

To quantitate the recombinant proteins used for EMSA assays, a dried Coomassie Blue stained gel of the recombinant proteins was initially scanned using a digital scanner (ScanMaker III) and the image stored. The image was then subjected to densitometry analysis using Fuji MacBAS V2.2 Phosphoimager program. The proteins were then diluted equally according to arbitrary densitometric units before use.

II.10: Preparation of crude cell extracts

Hela cells were grown in DMEM plus 10% fetal calf serum, while MO59J and MO59K cells grown in DMEM/F12 supplemented with 10% fetal calf serum (GIBCO-BRL) as described in Allalunis-Turner *et al.*, (1995). Jurkat T-cells were grown in RPMI medium with added 10% fetal calf serum as described in Corthesy and Kao (1994). High salt total protein cell extracts (HSE) were made as described in Allalunis-Turner *et al.*, (1995). Briefly, monolayer cells were gently scraped and resuspended in 15 mL of ice cold PBS (contents defined in Table II-1). Cells grown in suspension were pelleted by centrifugation at 1,500 x g at 4°C for 5 min., and resuspended in 15 mL of ice cold PBS. The resuspended cells were centrifuged at 1,500 x g at 4°C for 5 min., and the resulting cell pellet was resuspended in 15 mL of LSB (10 mM Hepes pH 7.2, 25 mM KCl, 10 mM NaCl, 1.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT). These resuspended cells were pelleted by centrifugation at 1,500 x g for 5 min., and gently resuspended in 100 µL of LSB (each extract contained approximately 10^6 cells). Cells in LSB were then frozen in liquid N_2 and thawed in the presence of 0.5 mM PMSF, and leupeptin and pepstatin at 2 µg/mL each. Extraction buffer (5 M NaCl, 100 mM $MgCl_2$, 10 mM DTT) was added to the thawed extract to a final concentration of 0.5 M NaCl, 10 mM $MgCl_2$, 1 mM DTT and incubated on ice for 5-10 min. These extracts were centrifuged at 10,000 x g for 2 min. at 4°C, to recover both cytoplasmic and nuclear proteins. The pellet was re-

extracted in the same buffer and the supernatant was combined with the first. Protein concentrations of the high salt cell extracts were determined by BioRad dye assays as described before, and were usually between 3.0 and 3.5 mg/mL. Cell extracts were stored in aliquots at -80°C for up to two weeks before use.

II.11: Antibody co-immunoprecipitation

A partially purified protein fraction from human placenta (termed pre-dsDNA cellulose) which contained approximately 20 μg of total proteins were incubated under standard EMSA assay conditions in the presence or absence of the 40 bp dsDNA (5 μg/mL). After the 10 min. of incubation, an equal volume of immunoprecipitation (IP) buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.1% Tween 20{v/v}) was added. These samples were pre-cleared with protein G-Sepharose (Pharmacia) followed by the addition of antibodies to DNA-PKcs, NF45, NF90, SV40 T-Ag, or pre-immune serum. They were then incubated with gentle rotation for 2 hours at 4°C and protein G-Sepharose was added to precipitate the immune-complexes. After the pellet was washed three times with 0.4 mL of IP buffer with 0.05% Tween 20 (v/v), the immune-complexes were analyzed by SDS-PAGE and Western blot as described above.

In immunoprecipitation experiments using crude Jurkat T-cell extracts, approximately 25 μg of total protein was used. The protocol used was as described above.

II.12: Immunodepletion experiments

An equal volume of immunoprecipitation (IP) buffer (as described before) was added to 5 μL of mouse pre-immune sera or mouse anti-sera to NF90 (different bleeds, NF90 ab.2 or ab.3), incubated with protein G-Sepharose beads (Parmacia-Biotech), and allowed to rotate at 4°C for 1 hour. The beads were then pelleted by centrifugation at 500 x g for 5 min. at 4°C. The pelleted beads were washed three times with 0.4 mL of IP buffer, and each time the beads were repelleted as described before. 20 μL of protein fraction (0.25-0.5 μg) was added to the antibody-coupled beads and rotated at 4°C for 1 hour. The samples were then spun at 500 x g for 5 min. and supernatants were removed and used either in EMSA or histone acetyltransferase (HAT) activity assays. Contents of

the supernatant were analyzed by Western immunoblot or silver-stained SDS-PAGE. The beads were further washed as before and resuspended in 4 x SDS sample buffer. Proteins bound to the beads were analyzed by SDS-PAGE and Western immunoblot.

II.13: Histone acetyltransferase assays

Histone acetyltransferase (HAT) filter binding activity assays were performed according to Brownell and Allis (1995). 0.1 - 0.2 μg of protein was incubated with 2 μg of calf thymus (CT) total histones (Sigma) and 1.0 μM acetyl-CoA (with 3.0 μCi ^{3}[H]-acetyl-CoA, {ICN Radiochemicals}) in buffer containing 50 mM KCl, 50 mM Tris-HCl pH 8.0, 10% glycerol, 10 mM sodium butyrate, 1.0 mM DTT for 30 min., at 30°C in a final reaction volume of 30 μL. The reactions were started by adding acetyl-CoA, and stopped by spotting the reaction mixture (in 15 μL duplicates) on 2 cm^2 squares of p81 phosphocellulose paper (Whatman). The squares were washed four times using 500 mL of 50 mM sodium bicarbonate, pH 9.2, for 15 min. each wash. The washed squares were allowed to air-dry for 30 min., and placed in 2.0 mL of scintillation fluid (EcoLume, ICN) to measure the amount of ^{3}H-acetate incorporation into the histones by scintillation counting.

For HAT assays using SDS-PAGE/autoradiographic analysis, the final reaction volume was 20 μL (with 2.0 μCi of ^{3}H-acetyl CoA) and the reactions were stopped by using 4 x SDS sample buffer. The reaction mixtures were fractionated on 15% acrylamide:0.2% bisacrylamide SDS gels, stained with Coomassie Blue and destained by standard methodology. The destained gel was subjected to fluorography by treatment with En^{3}Hance (Dupont-NEN) according to the supplier's instructions. After fluorography, the gel was dried and placed in cassettes with X-ray film (Fuji) and two intensifying screens at -80°C for at least 10 days before development.

II.14: In-Gel histone acetyltransferase assays

In-Gel HAT assays were performed according to Brownell and Allis (1995). Approximately 2 μg of protein sample was dissolved in 4 x SDS sample buffer at RT, but not boiled, and then loaded onto 10% acrylamide:0.2% bisacrylamide SDS gels that had been specially modified. This modification included dissolving total histones (from calf

thymus) to a final concentration of 1 mg/mL into the resolving gel prior to polymerization. Following electrophoresis, the gel was washed with gentle agitation for 4 times, 15 min. each, at RT using 100 mL of Buffer A (50 mM Tris-HCl pH 8.0, 1 mM DTT, 0.1 mM EDTA) containing 20% (v/v) isopropanol. Proteins in the gel were then denatured by incubating the gel in 100 mL of Buffer A containing 8 M urea for 1 hour at RT (4 washes, 15 min. each, gentle agitation). This was followed by two 15 min. washes with agitation at RT with 100 mL of buffer A containing 0.04% Tween 40 (Sigma). The proteins were allowed to renature by incubating the gel overnight at 4°C in 100 mL of the same buffer without agitation, followed by two more washes in the same buffer the next morning. The gel was allowed to reach RT and then incubated in Reaction Buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol (v/v), 1 mM DTT) for 30 min. Finally, the gel was then placed in a heat-sealable bag (8 x 10 cm) with 5 mL of the Reaction Buffer supplemented with 10 µCi of [^{3}H]-acetyl CoA. The bag was sealed and mixed thoroughly with care taken to immerse the gel with buffer and to exclude air-bubbles. Acetylation reactions were allowed to proceed for 1 hour at 30°C. The reactions were stopped by removing the gel and washing it with 250 mL of Reaction Buffer to remove unincorporated radioactivity. The gel was then stained with Coomassie Blue, destained and processed by fluorography and autoradiography as described above. As a control for non-specific incorporation of acetyl groups, BSA was polymerized into the gel instead of total histones.

CHAPTER III

RESULTS AND DISCUSSION - PART 1

IDENTIFICATION OF NOVEL SUBSTRATES OF DNA-PK

Chapter III - Identification of novel substrates of DNA-PK[1]

III.1: Introduction

During the initial isolation of DNA-PK from Hela cells, several polypeptides, that were present in a partially purified active kinase protein fraction, were shown to be phosphorylated in a DNA dependent manner (Lees-Miller *et al.*, 1990). Three of these polypeptides were identified as DNA-PKcs, Ku80 and Ku70. Two other polypeptides, which migrated on SDS gels with approximate molecular masses of 120 and 52 kDa, were also phosphorylated in the presence of calf thymus DNA (CT-DNA) and ATP (Figure 1 of Lees-Miller *et al.*, 1990). DNA-PKcs and the 120 and 52 kDa polypeptides were separated from Ku using a linear salt gradient on dsDNA-cellulose chromatography. DNA-PKcs was further purified away from the associated substrates using MonoQ-FPLC, followed by MonoS-FPLC in the presence of 0.02% Tween (Ting and Lees-Miller, unpublished results). Ku was also purified separately using the same FPLC strategy. Coomassie Blue stained gels and Western blot analysis showed that preparations of DNA-PKcs and Ku purified via this methodology were 98% pure. Full DNA-PK kinase activity required reconstitution of approximately equimolar amounts of DNA-PKcs and Ku (Gottlieb and Jackson, 1993; Suwa *et al.*, 1994; Chan *et al.*, 1996).

It is now established that Ku is an important DNA binding and regulatory component of DNA-PK. As Ku was first identified within this pool of phosphorylated polypeptides, it is therefore plausible that the other polypeptides in this pool might also interact and regulate DNA-PK activity. Furthermore, one of the proposed functions for DNA-PKcs is that it acts as a scaffolding protein, to recruit other proteins to sites of DNA damage. These polypeptides that were present in the partially purified DNA-PK fraction were therefore considered as possible candidates for proteins that may be recruited by DNA-PKcs. We therefore sought to purify and identify these polypeptides from Hela cells. However, yields for DNA-PK and the associated polypeptides from Hela cells were low. As an alternate source of DNA-PK, our laboratory developed methods to purify DNA-PK from human placenta. Although, rodent or bovine tissues were available, the human placenta tissue was chosen because DNA-PK is 50 to 100 fold more

[1] The results presented in this chapter have been published: Ting, N.S.Y., Kao, P.N., Chan, D.W., Lintott, L.G., and Lees-Miller, S.P. (1998) *DNA-dependent Protein Kinase Interacts with Antigen Receptor Response Element Binding Proteins NF90 and NF45.* J. Biol. Chem. 273(4):2136-2145.

abundant in humans compared to all other mammalian sources (Anderson and Lees-Miller, 1992; Finnie *et al.*, 1995; Danska *et al.*, 1996). Our laboratory has successfully purified DNA-PK to homogeneity from human placenta with relatively high yields (Chan *et al.*, 1996). Several polypeptides with approximately the same molecular masses as the substrates that were present in the active DNA-PK fraction from Hela cells were also present in partially purified preparations of DNA-PKcs from human placenta (Chan *et al.*, 1996). These polypeptides were therefore purified to determine if they were substrates of DNA-PK.

III.2: Results and Discussion

The method of purification for DNA-PKcs, Ku and associated polypeptides is outlined in Figure II-1, Chapter II (Materials and Methods). Briefly, approximately 200 g of frozen cubes of placenta tissue was homogenized in high salt buffer and precipitated with two different concentrations of ammonium sulfate. The pellet from the 60% ammonium sulfate precipitation step was resuspended and subjected to anion exchange chromatography using DEAE resin, followed by cation exchange chromatography using S-Sepharose resin. The eluent was then subjected to a second round of anion exchange chromatography (DEAE) in the presence of magnesium. Under these conditions, DNA-PKcs, Ku and several other polypeptides no longer bound to the DEAE resin and were present in the flow-through fractions. These fractions were pooled, and divided equally into three separate aliquots for application onto dsDNA-cellulose.

Polypeptides that bound to the dsDNA-cellulose resin were eluted with a linear gradient of salt from 0.1 M KCl to 1.0 M KCl at approximately 0.05 M KCl/min. The majority of the DNA-PKcs eluted between 0.1 and 0.3 M KCl and these fractions were referred to as pool A; Ku eluted between 0.3 and 1.0 M KCl and these fractions were designated as pool B (data not shown; Chan *et al.*, 1996). Several other polypeptides in addition to DNA-PKcs were present in pool A. One of these polypeptides migrated at a similar position in SDS gels as the 52 kDa substrate first described in Hela cells (data not shown; Lees-Miller *et al.*, 1990).

After all three aliquots of the Mg-DEAE flow-through sample were processed by dsDNA-cellulose gradient chromatography, fractions containing DNA-PKcs and the

associated polypeptides were combined (pool A). These combined fractions were dialyzed and subjected to chromatography using MonoQ-FPLC as described in Chapter II (Materials and Methods). The polypeptides that eluted with DNA-PKcs in pool A, eluted at a salt concentration of 0.25 to 0.3 M KCl, while the majority of DNA-PKcs eluted between 0.2 M and 0.25 M KCl. Optimal separation between DNA-PKcs and these polypeptides by MonoQ chromatography could only be achieved in the presence of a mild detergent (Tween 20), suggesting a possible physiological interaction between DNA-PKcs and these polypeptides. As seen in Figure III-1, these polypeptides included proteins of approximate molecular weights 90-, 75-, 52/50- , 45-, and 37 kDa (indicated as *p90, p75, p52/50, p45* and *p37*). The fractions containing these polypeptides were pooled and designated as pool C. In the experiment shown, the 52- and 50 kDa polypeptides migrated as at the same position on the 10% acrylamide SDS gel. Complete separation of the two polypeptides could be achieved using longer times of electrophoresis for the 10% acrylamide gel (to be discussed later).

Pool C was further purified using MonoS-FPLC, utilizing the protocol outlined in Chapter II (Materials and Methods). Silver staining of gels following SDS-PAGE analysis revealed that the same six polypeptides were present (indicated as *p90, p75, p50/52, p45 and p37, fractions 42-62*, Figure III-2) and these polypeptides eluted over a broad range of salt concentration (0.11 to 0.35 M KCl). Western blot and DNA-PK kinase assays indicated that these fractions were devoid of DNA-PKcs and Ku protein and activity, respectively (data not shown). In the 10% acrylamide SDS gel shown, a longer electrophoresis time was employed than that used for Figure III-1; therefore, p50/52 did not co-migrate, but migrated as two distinct polypeptides (*Fractions 48, 50, 52*; Figure III-2). Fractions 44 to 64 were pooled and dialyzed into the appropriate buffers as described in Chapter II (Materials and Methods). These combined fractions are hereafter referred to as the MonoS pool of polypeptides.

To determine which polypeptides within the MonoS pool were substrates for DNA-PK, proteins were incubated under phosphorylation assay conditions plus or minus DNA and processed by electrophoresis on 10% acrylamide SDS gels, followed by Coomassie Blue staining and autoradiography as outlined in Chapter II (Materials and Methods). Purified DNA-PK displayed characteristic DNA dependent auto-

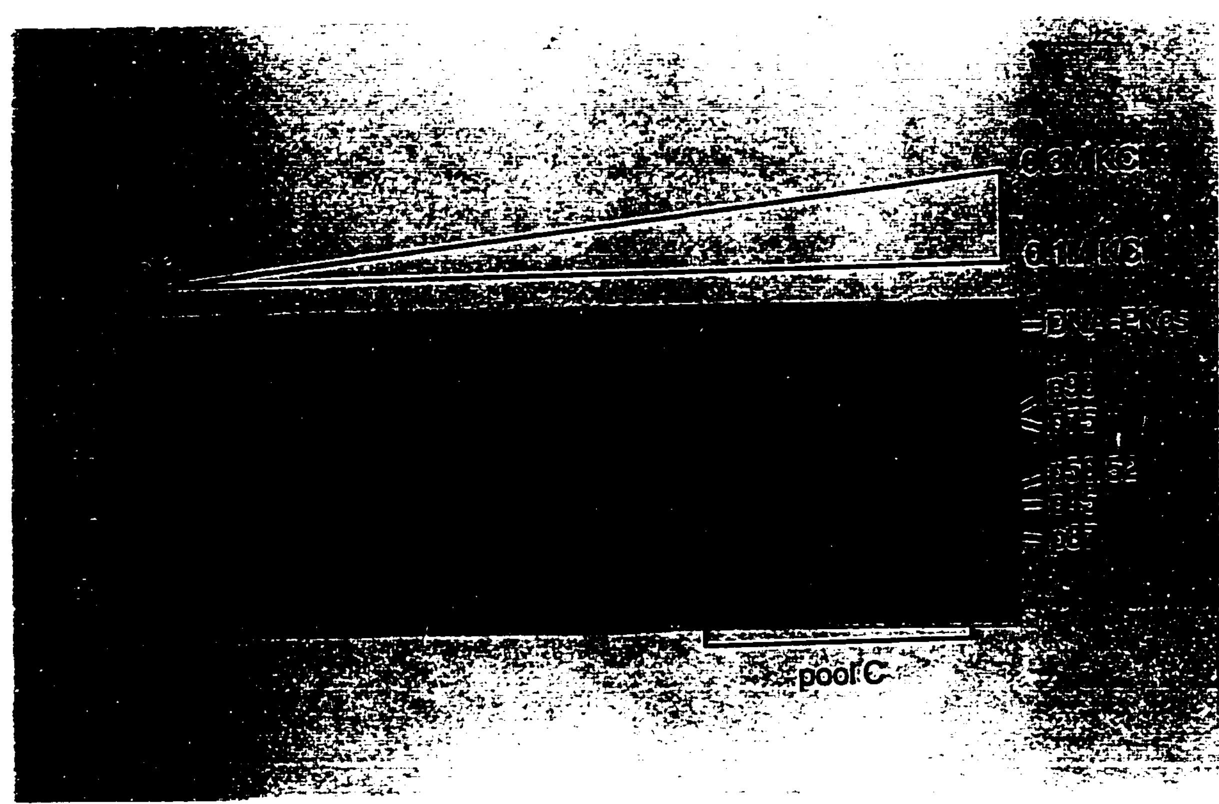

Figure III-1: MonoQ chromatography of pool A from dsDNA-cellulose gradient chromatography - Coomassie Blue stained SDS-PAGE. 10 µL of each fraction eluting from the MonoQ-FPLC between salt concentration range of 0.1 M and 0.3 M KCl was fractionated on 10% acrylamide SDS gel and stained with Coomassie Blue. MW markers in kDa are indicated on the left and the approximate molecular weights of the prominent polypeptides present are indicated on the right. These polypeptides were combined and referred to as pool C.

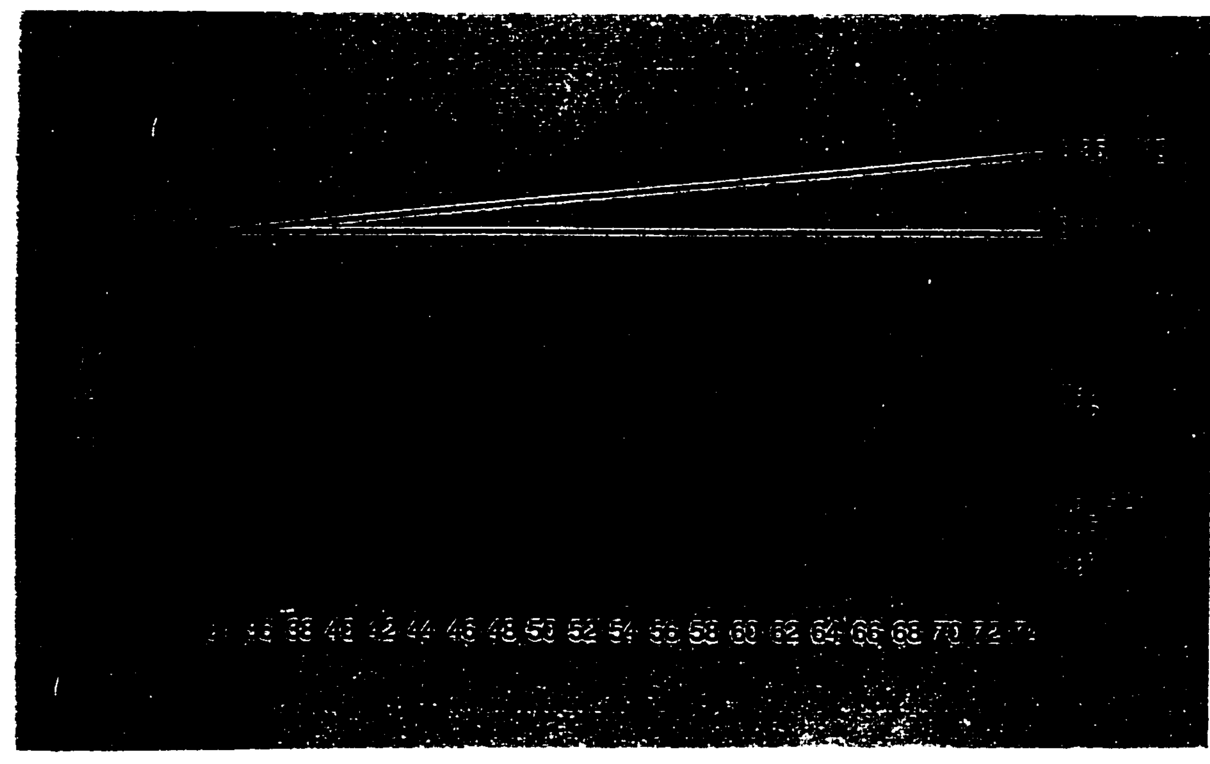

Figure III-2: MonoS chromatography of pool C from FPLC-MonoQ - silver-stained SDS-PAGE. 5 µL of even numbered fractions eluted between a salt concentration range of 0.11 M and 0.35 M KCl from the MonoS-FPLC were fractionated on 10% acrylamide SDS gel and silver stained. MW markers in kDa are indicated on the left and fraction numbers are indicated at the bottom of the panel. The polypeptides from pool C - p90. p75, p52, p50, p45 and p37 are indicated on the right. Fractions 44 to 64 were pooled together to from the MonoS pool. The letter X indicates a non-specific artifact of the silver staining process.

phosphorylation of DNA-PKcs and the Ku70/80 subunits (*lanes 1* and *2*, Figure III-3B). The MonoS pool of polypeptides assayed alone under the same conditions showed no endogenous phosphorylation (*lanes 3* and *4*, Figure III-3B). However, in the presence of DNA-PKcs and Ku70/80, the 90-, 50- and 45 kDa polypeptides were phosphorylated in a DNA dependent manner (Figure III-3B, *lanes 5* and *6*, indicated by *p90*, *p50/52* and *p45*). The two other polypeptides in the MonoS pool, p75 and p37, were not phosphorylated. Under the electrophoresis conditions utilized, p50 and p52 co-migrated in the MonoS pool (Figure III-3A , *lanes 3-6*). Therefore, similar samples were subjected to another round of electrophoresis for a longer time to separate the two polypeptides. The gel was silver stained and processed by autoradiography (Figure III-3C). Under these conditions, p52 separated from p50 (*lanes 1-4*, Figure III-3C), and p50 but not p52 was phosphorylated by DNA-PK (Figure III-3D, *lanes 3* and *4*). It is interesting to note that autophosphorylation of DNA-PK diminished slightly in the presence of the MonoS pool of polypeptides (Figure III-3B, compare *lane 5* to *lane 1*). It has been shown that the presence of substrates prevents DNA-PK autophosphorylation and inactivation of kinase activity (Chan and Lees-Miller, 1996). In this case, p90, p50 and p45 are substrates that reduced the level of DNA-PK autophosphorylation.

To obtain amino acid sequence information for these polypeptides, the MonoS pools from 3 separate placenta preparations were combined to yield approximately 20 μg of each polypeptide. The MonoS pool was fractionated on a 10% acrylamide SDS gel, transferred onto sequencing grade PVDF (polyvinylidene diflouride) membrane and stained briefly with Coomassie Blue. The membrane was destained, washed with water and allowed to air-dry. The 75-, 45-, 52/50- and 37 kDa polypeptides were excised and sent for N-terminal amino acid sequence analysis as described in Chapter II (Materials and Methods). In the sample sent for sequencing, p52 and p50 were not resolved and therefore both polypeptides were subjected to N-terminal amino acid sequence analysis in the same sample. In the combined MonoS pool sample, p75 was present in greater abundance than p90, and therefore only the p75 polypeptide band was excised for amino acid sequence analysis. The results obtained are summarized in Table III-1.

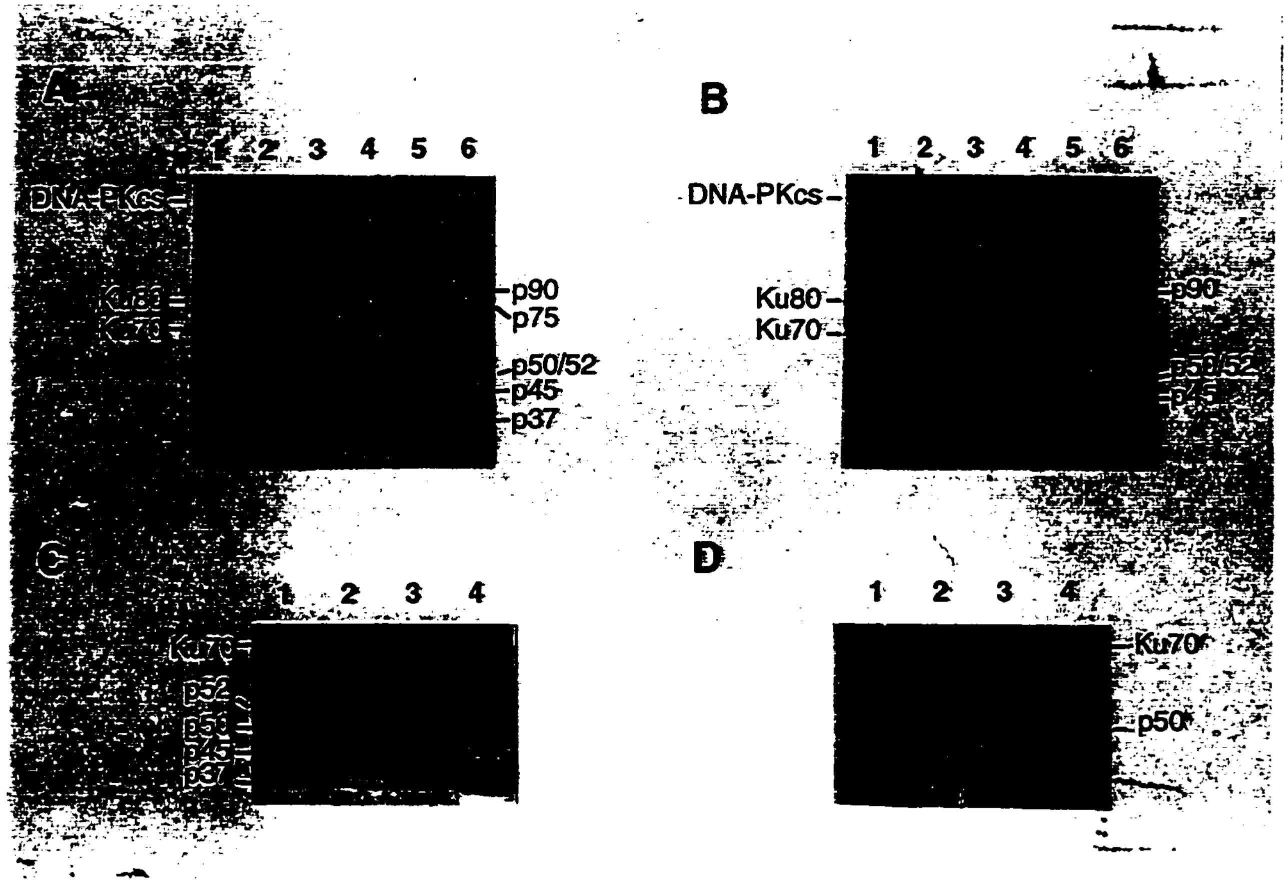

Figure III-3: Phosphorylation of p50, p45, p90 by DNA-PK *in vitro*. Phosphorylation reactions were carried out as indicated in Chapter II (Materials and Methods) and the products were analyzed by 10% acrylamide SDS gel, Coomassie Blue staining and autoradiography.

A) 10% acrylamide SDS gel - Coomassie Blue stained and **B) Corresponding autoradiogram.** The contents of each phosphorylation reaction are as follows: *lanes 1* and *2* - DNA-PK +/- DNA; *lanes 3* and *4* - MonoS pool +/- DNA; *lanes 5* and *6* - DNA-PK plus MonoS pool +/- DNA. Phosphorylated polypeptides p90, p50 and p45 are indicated on the right hand side of panel B.

C) A portion of the 10% acrylamide SDS gel - silver-stained and **D) Corresponding autoradiogram.** Similar reactions were subjected to another round of electrophoresis and the contents of each reaction are as follows: *lanes 1* and *2*- MonoS pool +/- DNA; *lanes 3* and *4* -DNA-PK plus MonoS pool +/- DNA. Phosphorylated Ku70 and p50 are indicated on the right side of panel D.

Table III-1: N-terminal amino-acid sequence analysis of the polypeptides in the MonoS pool.

Once the MonoS pool was transferred onto sequencing grade PVDF membrane as described in Chapter II (Methods and Material), appropriate protein bands were excised and sent for N-terminal amino acid sequence analysis. Amino acid sequence results are expressed as single letter codes; upper case letters represent a definitive match, while lower case letters represent ambiguous amino acid assignments. Letter X represents an unknown amino acid. Shown below are the results obtained and the corresponding matching N-terminal amino acid sequence of known gene products. They included NF90 (GenBank accession no., U10324); NF45 (GenBank accession no., U10323); eIF-2α (GenBank accession no., J02645) and eIF-2γ (GenBank accession no., L19161). The full amino acid sequence of each polypeptide is presented in Chapter X, Appendix - Figure X-1 and X-2.

MonoS pool polypeptide	N-terminal sequence obtained	Matching sequence in GenBank	Identity of polypeptide
p75	MRPMRIFVND	MRPMRIFVND	NF90
p52/50	AGGEAGVtLgQPHLSR	MAGGEAGVTLGQPHLSR	eIF-2γ
p45	MRGDRGRGRG	MRGDRGRGRG	NF45
p37	p/tGLs/aXr/tFYQHKEPE	MPGLSCRFYQHKFPE	eIF-2α

The N-terminal amino acid sequences obtained for all four excised protein bands matched those of known gene products (Table III-1). Approximately 90% of the first 16 amino acids read for p52/50 corresponded to the N-terminal amino acid sequence of eIF-2γ; while, 85% of the first 14 amino acids obtained for p37 aligned with the amino terminus of eIF-2α (Table III-1). In the case of the remaining 10% and 15% of the amino acids obtained for p52/50 and p37, respectively, it was not possible to unambiguously identify the amino acids present. The eukaryotic translation Initiation Factor, eIF-2, is a heterotrimeric protein of α, β, and γ subunits with molecular weights of 37, 38, and 52 kDa, respectively, as predicted from their cDNA sequences. eIF-2 associates with GTP to bind initiator methionyl tRNA (Met-tRNAi) and the 40S ribosomal subunit to form a ternary complex. In turn this complex recruits the 60S ribosomal subunit and mRNA to initiate translation. Upon binding of the 60S subunit, GTP is hydrolyzed, and the eIF-2-GDP complex is released. Regeneration of eIF-2-GTP is required to initiate another round of translation (reviewed in Hershey, 1991; Merrick and Hershey, 1996).

The amino-terminal sequence obtained for p75 and p45 were a perfect match with polypeptides called NF90 and NF45 (Table III-1). NF90 and NF45 are novel nuclear proteins that were initially identified by their ability to bind the antigen receptor response element (ARRE) of the interleukin-2 (IL-2) promoter region, in Jurkat T-cells that had been stimulated with phorbol myristate acetate (PMA) and ionomycin. Therefore, NF90 and NF45 were initially proposed to be a heterodimeric transcription factor responsible for the expression of IL-2 upon activation of T lymphocytes (Kao and Corthesy, 1994; Kao *et al.*, 1994).

The presence of these polypeptides in the MonoS pool was confirmed by Western immunoblot analysis of fractions eluted from FPLC-MonoS chromatography (Figure III-4). Rabbit polyclonal antibodies to recombinant NF90 and NF45 cross reacted with p90 and p75 (Figure III-4A, *fractions 46-58*), and p45, respectively (Figure III-4B, *fractions 46-58*, antibodies were a kind gift from Dr. Peter Kao, Stanford University). p50 and p52 cross-reacted with rabbit polyclonal antibodies to purified human eIF-2β and 2γ, respectively (Figure III-4C, *fractions 46-56*, and data not shown; antibodies were kind gifts from Dr. John Hershey, UC Davis).

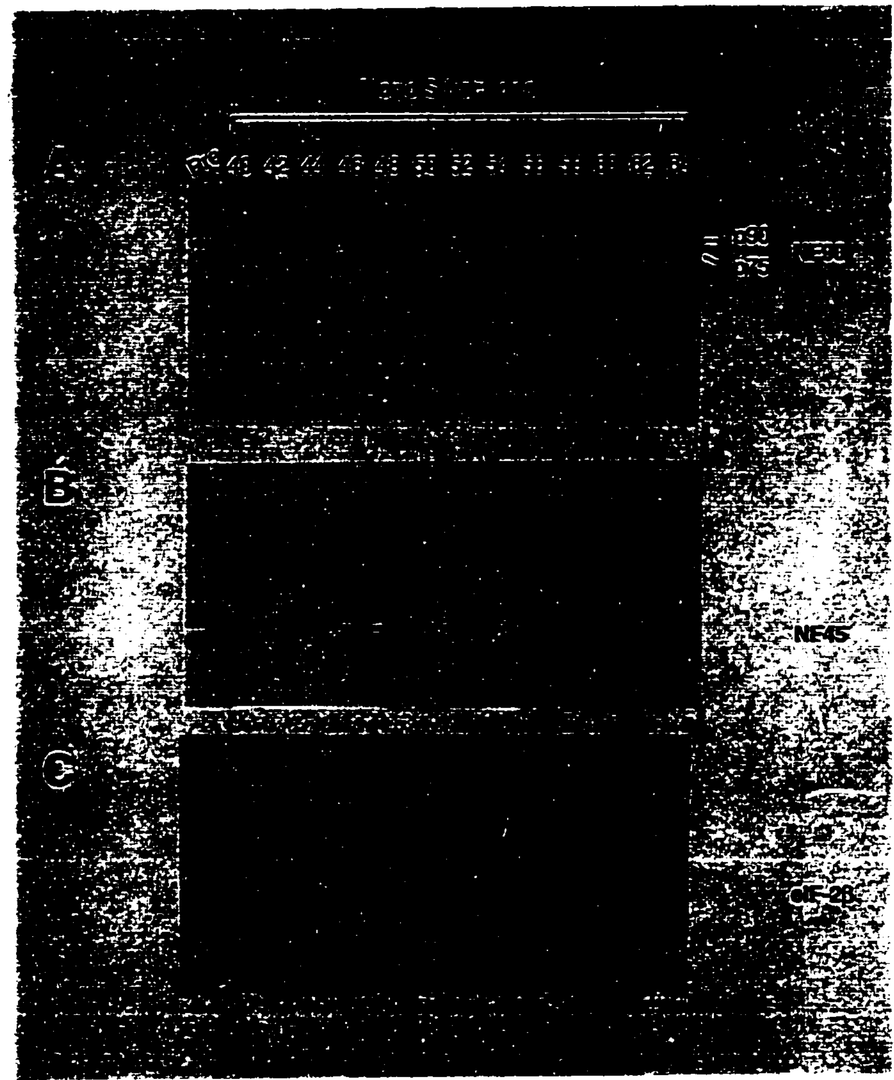

Figure III-4: Western blot analysis of the MonoS-FPLC fractions with antibodies to NF90, NF45 and eIF-2β. 10 μL of even numbered fractions eluted from the MonoS-FPLC column were fractionated on 10% acrylamide SDS gel and transferred to PVDF membrane according to the procedure outlined in Chapter II (Materials and Methods). The membrane was analyzed using antibodies to **A)NF90**, **B)NF45** and **C)eIF-2β**. The identity of each polypeptide is indicated on the right side of each panel.

Since the N-terminal amino acid sequence obtained for p75 matched perfectly with NF90 and since it cross-reacted with antibodies to NF90, we speculate that p75 may be a proteolytic breakdown product of NF90. Consistent with this notion, p75 and p90 were co-immunoprecipitated with antibodies to NF90 (Chapter V, Figure V-7). On the other hand, during the initial cloning of NF90, two cDNA clones of different sizes were isolated for NF90. The larger clone was predicted to encode an open reading frame of 671 amino acids, while the smaller clone was predicted to encode an open reading frame of 404 amino acids with the first 394 amino acids identical to the larger clone. *In vitro* translation of these clones yielded polypeptides that migrated as 90 and 55 kDa, respectively, on denaturing gels (Kao *et al.*, 1994). The larger clone was therefore considered to be the cDNA sequence for NF90. Kao and colleagues concluded that the smaller clone was related to the larger clone but represented a different gene product (Kao *et al.*, 1994). This suggested to us that p75 could potentially be a variant form of NF90. Since p90 was phosphorylated by DNA-PK *in vitro*, and p75 was not (Figure III-3), we speculate that p75 may lack a C-terminal 15 kDa region that contains the DNA-PK phosphorylation sites. Purified bacterially expressed recombinant NF90 and NF45 (rNF90/rNF45) were also phosphorylated by DNA-PK *in vitro* (data not shown). To date, DNA-PK is the first kinase shown to phosphorylate the heterodimeric transcription factors NF90 and NF45. Addition of phosphatase to nuclear extracts from activated Jurkat T-cells containing NF90 and NF45, reduced binding to the ARRE element (Kao *et al.*, 1994). These data suggest that NF90 and NF45 are phosphoproteins and their function may be regulated by phosphorylation *in vivo* (Kao and Corthesy, 1994; Kao *et al.*, 1994). From these data, we conclude that NF90 and NF45, which are present as p90 and p45 in the MonoS pool, are novel *in vitro* substrates of DNA-PK. We also show that p75, a polypeptide that is related to NF90, is not a substrate for DNA-PK.

The other polypeptides present in the MonoS pool were identified as the heterotrimeric translation initiation factor, eIF-2. Four lines of evidence suggest that the p50 polypeptide in the MonoS pool is eIF-2β and it is an *in vitro* substrate of DNA-PK. First, it is known that eIF-2β does not migrate on SDS-PAGE according to its predicted molecular weight (Pathak *et al.*, 1988). In fact, it often migrates with the 52 kDa subunit (eIF-2γ), depending on the SDS-PAGE conditions employed (Wettenhall *et al.*, 1986;

Pathak *et al.*, 1988). This is also consistent with our observations, in that varying the electrophoresis time altered the migration of p50 relative to that of p52. Secondly, the N-terminus of eIF-2β is blocked (Pathak *et al.*, 1988). This provides an explanation for why the only sequence we obtained for the p52/50 polypeptides matched with eIF-2γ (Table III-1). Thirdly, antibodies to eIF-2β cross reacted specifically with p50 (Figure III-6C). Finally, phosphorylated p50 migrated on two-dimensional gel electrophoresis close to actin, which has a pI of 5.5 (data not shown), consistent with the known behavior of eIF-2β, which has a pI of 5.9 (Pathak *et al.*, 1988; Gaspar *et al.*, 1994). As further evidence that p50 is eIF-2β, we obtained purified rat liver eIF-2 (a kind gift from Dr. Scott Kimball, Pennsylvania State University). A polypeptide corresponding to eIF-2β in the rat liver eIF-2 preparation was phosphorylated in the presence of DNA-PK, CT-DNA and $^{32}\gamma$P-ATP (data not shown). Taken together, these data strongly suggest that p50 in the MonoS pool is eIF-2β and that it is an *in vitro* substrate of DNA-PK.

Since DNA-PK is predominantly a nuclear kinase, this led us to question why it should phosphorylate a cytoplasmic factor involved in translation. eIF-2α is phosphorylated by several protein kinases (including the dsRNA dependent protein kinase [PKR]) and phosphorylation leads to inhibition of protein synthesis (reviewed in Hershey, 1989; 1991). eIF-2β is phosphorylated by Casein Kinase II and Protein Kinase C *in vitro*, but the consequence of phosphorylation is unknown (Flynn *et al.*, 1993). We observed that eIF-2, existing in the MonoS pool as p52/50 and p37, was able to bind dsDNA and ssDNA-cellulose resins during the purification from human placenta. Examination of the amino acid sequence of eIF-2β revealed the existence of poly-lysine regions that are capable of binding nucleic acids (Pathak *et al.*, 1988; see Figure X-1, Chapter X). eIF-2β has been shown to be required for binding of eIF-2 to mRNA (Flynn *et al.*, 1994); however, whether the ability of eIF-2 to bind nucleic acids has any relevance *in vivo* remains to be determined. It is also possible that these strings of positively charged lysine residues act as nuclear localization signals. Consistent with this possibility, Lobo and colleagues recently showed, using immunofluorescence staining, that eIF-2 is localized to the nucleus as well as the cytoplasm (Lobo *et al.*, 1997). However, these studies were done only with monoclonal antibodies to eIF-2α, and eIF-

2β may behave differently from eIF-2α. Nevertheless, this data suggest that eIF-2 may have other roles in addition to its role in translation initiation.

To date, eIF-2 is not the only cytoplasmic factor found to be phosphorylated by DNA-PK *in vitro*. Others include the 90 kDa heat shock protein, hsp90, and the cytoskeletal protein, Tau (Table I-1, Chapter I; reviewed in Anderson, 1993). Interestingly, recent reports suggest that other members of the PI-3 related kinase family may have a role in regulating protein synthesis. ATM was recently shown to phosphorylate PHAS-1 (or eIF-4E binding protein-1) *in vitro* (Banin *et al.*, 1998). Phosphorylation of PHAS-1 (or 4E-BP1) has been shown to be required for the initiation of translation in response to mitogenic signals (Lin *et al.*, 1994; Pause *et al.*, 1994). Moreover, in budding yeast, inhibition of TOR1 and TOR2 resulted in reduced translation rates causing G1 growth arrest (Barbet *et al.*, 1996). Hence, it is possible that phosphorylation of eIF-2 by DNA-PK may have some physiological relevance.

III.3: Summary

We have identified three *in vitro* substrates of DNA-PK that partially copurified with DNA-PKcs from human placenta. These substrates are the translation initiation factor eIF-2, and transcription factors, NF45 and NF90.

CHAPTER IV

RESULTS AND DISCUSSION - PART 2

STABILIZATION OF DNA-PK$_{CS}$ AND KU ON DNA IN ELECTROPHORETIC MOBILITY SHIFT ASSAYS REQUIRES PROTEINS PRESENT IN THE MONO-S POOL

CHAPTER IV - Stabilization of DNA-PKcs and Ku on DNA in electrophoretic mobility shift assays requires proteins present in the MonoS pool[2]

IV.1: Introduction

We have shown that NF90/45 and eIF-2β are *in vitro* substrates of DNA-PK. Since NF90/45 and eIF-2 were found to be present in a partially purified active DNA-PK fraction along with DNA-PKcs, we wanted to address whether or not NF90/45 or eIF-2 interacted with DNA-PK and influenced its kinase activity. Furthermore, since DNA-PK is involved in non-homologous end-joining (NHEJ), it was important to determine if NF90/45 or eIF-2 might affect the interaction between DNA-PKcs and Ku on ends of dsDNA. The interaction of Ku on DNA has been extensively studied using EMSA (Mimori and Hardin, 1986; de Vries *et al.*, 1989; Blier *et al.*, 1993; Bliss and Lane, 1997). Results from these studies have led to a model describing the binding of Ku to DNA. Ku binds to the ends of DNA and translocates to internal sites of the DNA in an energy independent manner. Depending on the amount of Ku and length of DNA, multiple Ku molecules can bind to the same piece of DNA. This process can be visualized as a ladder of protein-DNA complexes in EMSA studies (reviewed in Dynan and Yoo, 1998). However, very little is known about the interaction between DNA-PKcs and the Ku-DNA complex. Our laboratory has developed an EMSA strategy to study the interaction between DNA-PKcs, Ku and DNA. The effects of NF90/45 and eIF-2 on the interaction between DNA-PK and DNA were therefore assessed.

IV.2: Results and Discussion

Titration of increasing amounts of the MonoS pool of proteins, which contained NF90/45 and eIF-2, into kinase reactions containing equimolar amounts of purified DNA-PKcs and Ku had no effect on DNA-PK activity when assayed in synthetic peptide assays (data not shown). This result suggests that NF90/45 and eIF-2 do not affect kinase activity *in vitro*.

We next used EMSA to study the interaction between DNA-PKcs and Ku. The

[2] All of the results presented in this chapter have been published: Ting, N.S.Y., Kao, P.N., Chan, D.W., Lintott, L.G., and Lees-Miller, S.P. (1998) *DNA-dependent Protein Kinase Interacts with Antigen Receptor Response Element Binding Proteins NF90 and NF45.* J. Biol. Chem. 273(4):2136-2145.

conditions of the EMSA used are outlined in Chapter II (Materials and Methods). Equimolar amounts of both strands of a 40 bp synthetic oligonucleotide were annealed and labeled with γ-^{32}P-ATP to create a blunt ended 40 bp duplex DNA probe containing no known specific DNA binding sequences. When the 40 bp DNA probe was incubated with highly purified Ku heterodimer under the specified assay conditions, two protein-DNA complexes were formed (labeled *a* and *b* in Figure IV-1, *lane 2*). Similar protein-DNA complexes have been observed by several groups (Mimori and Hardin, 1986; Paillard and Strauss, 1991; Blier *et al.*, 1993; Bliss and Lane, 1997). Although their exact composition is not known, it has been suggested that complexes *a* and *b* represent Ku binding to both ends and internal sequences of DNA or to the formation of Ku multimers on DNA (Mimori and Hardin, 1986; deVries *et al.*, 1989; Paillard and Strauss, 1991; Blier *et al.*, 1993).

Under the EMSA conditions used, highly purified DNA-PKcs showed no interaction with the 40 bp DNA probe (Figure IV-1, *lane 3*). Titration of up to a 1.3 fold molar excess of highly purified DNA-PKcs into reactions containing Ku and the 40 bp DNA probe did not alter the migration of the Ku-DNA complexes (Figure IV-1, *lanes 4 to 7*); addition of DNA-PKcs up to a 3 fold molar excess also had no effect on the Ku-DNA complexes (data not shown). Although the interaction of Ku and DNA-PKcs on DNA has been previously shown by antibody co-immunoprecipitation, UV cross-linking and DNAase footprinting studies (reviewed in Lees-Miller, 1996), under our EMSA conditions, there was no indication that DNA-PKcs interacted with Ku on the 40 bp DNA probe. Varying the concentration of NaCl (50 to 150 mM), DTT (1 to 10 mM), EDTA (0.1 to 0.5 mM) or $MgCl_2$ (0 to 5 mM) had no effect on the *a* and *b* complexes in EMSA reactions containing DNA-PKcs, Ku and the 40 bp DNA probe (data not shown).

Previous studies have shown that the addition of chemical cross-linkers such as glutaraldehyde enhanced the formation of a protein-DNA complex between p53 and its cognate DNA sequence (Wang *et al.*, 1995). We therefore asked whether or not chemical cross-linkers had an effect on the interaction between DNA-PKcs and Ku on DNA. Glutaraldehyde was added to a final concentration of 0.06% (v/v) into our EMSA reaction mixtures. Protein samples were incubated with the 40 bp DNA probe for 10

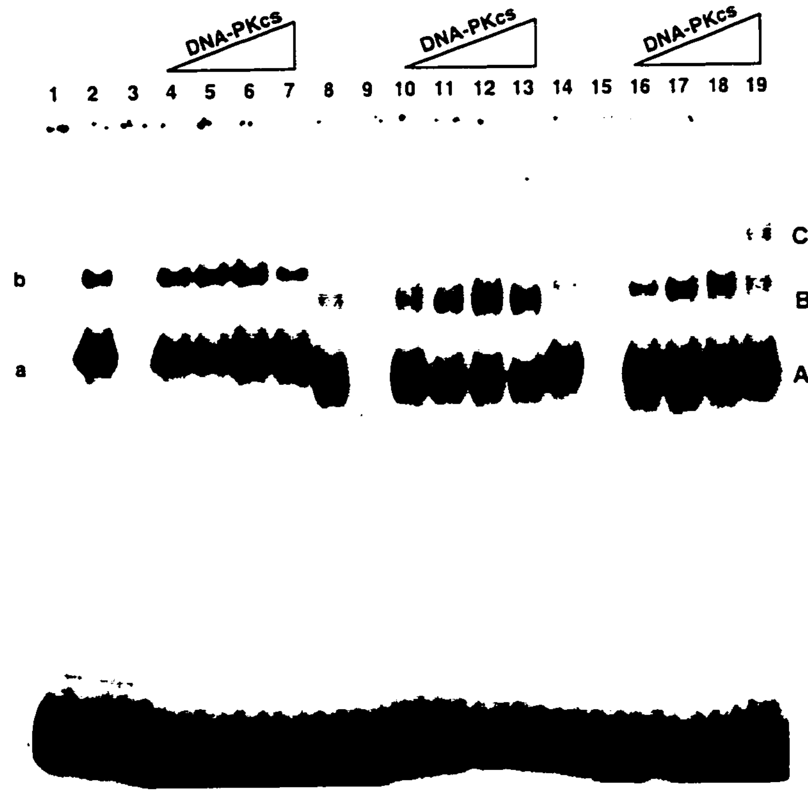

Figure IV-1: EMSA studies with DNA-PKcs, Ku, 40 bp DNA probe and chemical cross-linkers. The protocol and assay conditions used were as described in Chapter II (Materials and Methods). The amount of Ku used was 5 ng, the final concentration of glutaraldehyde and BS^3 used were 0.06% (v/v) and 1.0 mM, respectively. All reactions were initially incubated for 10 min. at RT and subjected to analysis by non-denaturing electrophoresis and autoradiography. Reactions with chemical cross-linkers were incubated for an additional 10 min. after the addition of cross-linkers before electrophoresis. The contents of each lane are as follows: *lane 1* - 40 bp probe alone; *lanes 2, 8, 14* - 5 ng Ku; *lanes 3, 9, 15* - 10 ng DNA-PKcs; *lanes 4-7, 10-13* and *16-19* - 5 ng Ku plus 10, 20, 30 and 50 ng of DNA-PKcs, respectively. Samples in *lanes 8-14* were treated with glutaraldehyde, while samples in *lanes 14-19* were treated with BS^3 prior to electrophoresis. Ku-DNA complexes formed in the absence of cross-linkers are indicated with lower case *a* and *b*, while Ku-DNA complexes formed in the presence of chemical cross-linkers are designated with upper case *A* and *B*. The much slower migrating band indicated by upper case *C* represent the putative DNA-PKcs-Ku-DNA complex.

min., before being treated with glutaraldehyde. These reactions were incubated for another 10 min. and analyzed by non-denaturing gel electrophoresis. In the presence of glutaraldehyde, the Ku-DNA complexes showed slightly increased mobility (labeled *A* and *B* in Figure IV-1, compare *lane 2* to *lane 8*). Faster migration of protein-DNA complexes treated with glutaraldehyde in EMSA has been previously observed and was attributed to the formation of a more compact protein-DNA structure (Wang *et al.*, 1995). Addition of glutaraldehyde to reactions containing Ku and increasing amounts of DNA-PKcs resulted in the formation of a slower migrating complex labeled *C* (Figure IV-1, *lanes 10-13*). The signal for the radiolabeled 40 bp DNA probe within this complex appeared as a discrete band and increased in intensity with increasing amounts of DNA-PKcs (Figure IV-1, *lanes 10-13*).

Since glutaraldehyde may promote non-specific cross-linking between proteins and between proteins and DNA, we tested another chemical cross-linker BS^3 (Bis[sulfosuccinimidyl] suberate). BS^3 is a non-cleavable thiol cross-linker that targets free amino groups over a distance of 11Å. Addition of BS^3 to a final concentration of 1 mM also resulted in the formation of complex *C*, which was similar in probe signal intensity to that seen with glutaraldehyde treatment (Figure IV-1, *lanes 16-20*). Neither glutaraldehyde nor BS^3 promoted or stabilized an interaction between DNA-PKcs and the 40 bp DNA probe (Figure IV-1, *lanes 9* and *15*), consistent with previous observations that DNA-PKcs does not interact with DNA in the absence of Ku (Gottlieb and Jackson, 1993; Suwa *et al.*, 1994). No other protein-DNA complexes were formed between heat denatured DNA-PKcs, Ku and DNA or between BSA, Ku and DNA in the presence of chemical cross-linkers (data not shown), suggesting that formation of complex *C* is dependent on the specific interaction between DNA-PKcs, Ku and DNA. Furthermore, formation of complex *C* was reduced in the presence of excess unlabeled 40 bp DNA, but not with closed-circular plasmid DNA (data not shown). These results are consistent with what is known about the DNA binding behavior of Ku and of Ku containing protein-DNA complexes (Paillard and Strauss, 1991; Blier *et al.*, 1993; Bliss and Lane, 1997). Taken together, these data suggest that BS^3 and glutaraldehyde promoted or stabilized the specific interaction between DNA-PKcs and Ku on DNA.

The formation of the putative DNA-PKcs-Ku-DNA complex in the presence of

chemical cross-linkers could be attributed to a number of possibilities. Firstly, the interaction between highly purified DNA-PKcs and Ku on DNA may be transient or unstable, with very high dissociation/association rates. In the presence of chemical cross-linkers, the proteins may become covalently associated with each other, consequently stabilizing the interaction and forming a distinct protein-DNA complex in EMSA. Secondly, the predicted total molecular weight of a protein-DNA complex containing DNA-PKcs, Ku, and the 40 bp DNA probe is approximately 650 kDa. Under our EMSA conditions, this complex may be too large to migrate into the gel by electrophoresis. Treatment of the complex with chemical cross-linkers made it more compact, enabling resolution on our non-denaturing gel system. If this were true, protein-DNA complexes would be retained in the base of the well and radioactivity would be detected at the top of the gel. However, this was not usually the case in untreated DNA-PKcs/Ku/DNA samples (Figure IV-1, *lanes 4-7*). Any protein-DNA complexes that did form within these samples were fractionated by electrophoresis (Figure IV-1, *lanes 4-7*). These data suggest that the chemical cross-linkers act primarily by stabilizing a protein-DNA complex containing DNA-PKcs and Ku. These data also suggested to us that this EMSA strategy could be used to assay for other factors that could stabilize the transient interaction between DNA-PKcs and Ku on DNA. Since NF90, NF45 and eIF-2 partially co-purified with DNA-PKcs through several chromatographic columns, we tested the possibility, whether or not these proteins affected the interaction between DNA-PK and DNA.

Individual aliquots of fractions 42 to 70 from MonoS-FPLC which contained NF90/45 and eIF-2, were incubated with purified DNA-PKcs, Ku and the 40 bp DNA probe in the **absence** of chemical cross-linkers and analyzed by EMSA. These samples produced a protein-DNA complex that migrated as a "discrete band" in a similar position to the previously described complex *C*. This complex is labeled *D* in Figure IV-2 (*lanes 13-17*). Significantly, complex *D* was formed at the expense of the Ku-DNA complexes *a* and *b* (Figure IV-2, *lanes 13-17*). The polypeptides in the MonoS-FPLC fractions by themselves did not support the formation of any protein-DNA complexes (Figure IV-2, *lanes 2-9*), although some retardation of the DNA probe was observed (Figure IV-2, *lanes 4-6*). Addition of the MonoS-FPLC fractions to Ku alone did not result in the

Figure IV-2: EMSA studies with MonoS-FPLC fractions, Ku, DNA-PKcs and the 40 bp DNA probe. Approximately 50 ng of total protein from every fourth fraction starting from 42 to 70, eluted from the MonoS FPLC column were incubated with probe alone or with DNA-PKcs (30 ng) plus Ku (10 ng) and probe in the **absence** of chemical cross-linkers and analyzed by EMSA as described in Chapter II (Materials and Methods). The contents of each lane are as follows: *Lane 1* - probe alone; *lanes 2-9* - MonoS fractions alone; *lane 10* - Ku alone; *lane 11* - Ku plus DNA-PKcs; and *lanes 12-19* - DNA-PKcs, Ku plus the MonoS fractions. The Ku-DNA complexes are indicated as *a* and *b*; the new complex formed in the presence of the MonoS fractions and DNA-PKcs plus Ku is indicated by upper case *D*.

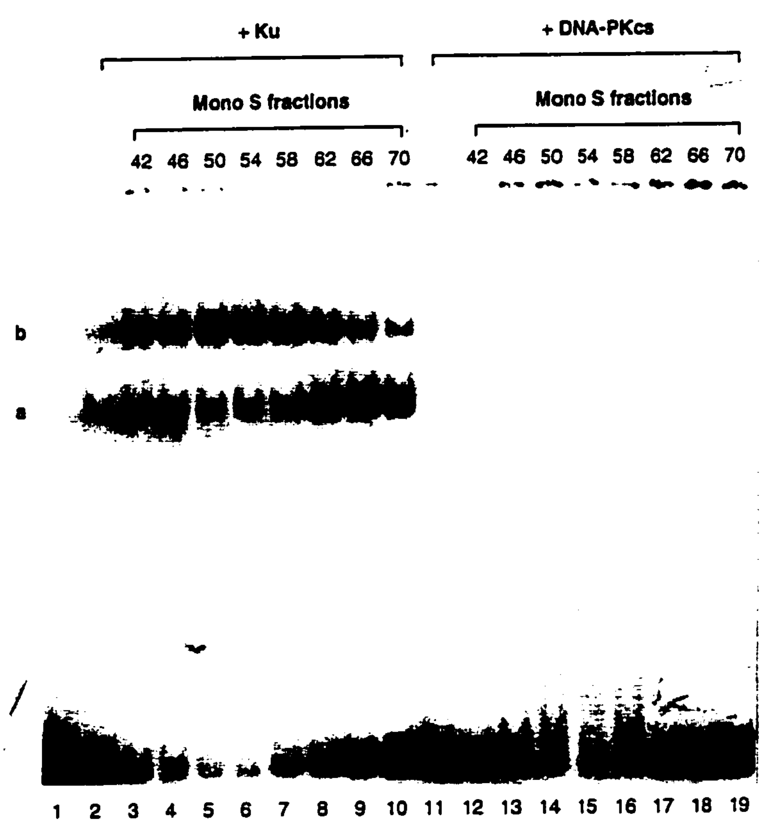

Figure IV-3: EMSA studies with MonoS-FPLC fractions incubated with Ku or DNA-PKcs and the 40 bp DNA probe. The MonoS fractions were incubated with probe plus Ku alone or with DNA-PKcs alone and the mixtures were analyzed by EMSA as before. The contents of each lane are as follows: *Lane 1* - probe alone; lane 2 - Ku alone; *lanes 3-10* - MonoS fractions plus Ku; *lane 11* - DNA-PKcs alone; and *lanes 12-19* - MonoS fractions plus DNA-PKcs. The Ku-DNA complexes are indicated as *a* and *b*.

formation of any new protein-DNA complexes (Figure IV-3, *lanes 3-10*), although certain fractions did promote an enhancement of the Ku-DNA complex *b* (Figure IV-3, *lanes 5-8*). This suggested that these polypeptides may induce a change in the interaction of Ku with DNA. However, since the nature of the Ku protein-DNA complexes *a* and *b* is not known, the significance of this observation is presently unknown and requires further investigation. Lastly, electrophoresis of samples containing MonoS-FPLC fractions and DNA-PKcs alone (plus the 40 bp DNA probe) showed no formation of protein-DNA complexes (Figure IV-3, *lanes 12-19*), indicating that the polypeptides in the MonoS fractions do not promote any DNA-PKcs-DNA complexes, and that Ku is required for the formation of complex *D*. In short, these data suggested that the polypeptides in fractions from MonoS-FPLC which contains NF90/45 and eIF-2 behaved similarly to chemical cross-linkers in that they were able to promote a stabilized complex between DNA-PKcs and Ku on DNA.

To ensure that the observed complex *D* was due to specific protein-protein interactions, and not due to fortuitous changes in the assay conditions, such as changes in the salt concentration or pH, fractions 44 to 64 eluting from the MonoS-FPLC were pooled (referred to as the MonoS pool) and control experiments were performed in which the MonoS pool was applied to a microconcentrator, Centricon 100 (Amicon) (Figure IV-4). This apparatus acts as a spin column in that it contains a membrane which retains molecules greater than 100 kDa in size, while allowing smaller molecules to pass through the membrane when the apparatus is subjected to centrifugation. The MonoS sample retained in the microconcentrator promoted the formation of complex *D* to an extent similar to that seen with the untreated MonoS pool sample (compare *lanes 10* and *11* to *lanes 8* and *9*, Figure IV-4), while the sample that flowed through the membrane did not promote the formation of complex *D* (Figure IV-4, *lanes 12* and *13*). This suggested that proteins greater than 100 kDa in size were responsible for the observed "stabilizing activity" in the MonoS pool. Furthermore, boiling the MonoS pool prior to the EMSA reaction abolished the "stabilizing activity" (Figure IV-4, *lanes 14* and *15*), while addition of BSA or ovalbumin, at two different concentrations, did not induce the formation of any new protein-DNA complexes (Figure IV-3, *lanes 17, 18* and *lanes 19, 20,* respectively). Consistent with previous observations, the MonoS pool did not form

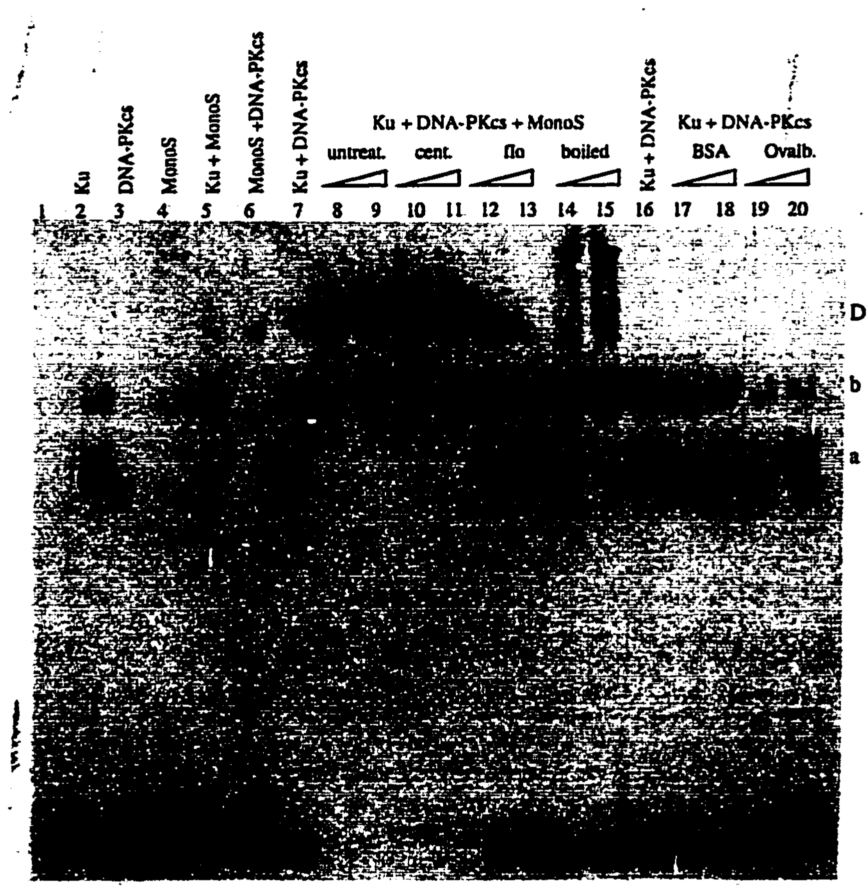

Figure IV-4: Control EMSA studies with MonoS pool, DNA-PKcs, Ku and the 40 bp DNA probe. Fractions 44 to 64 eluted from the MonoS-FPLC were pooled to form the MonoS pool of polypeptides. Approximately 60 ng of the MonoS pool was incubated with probe alone, with Ku (10 ng), with DNA-PKcs (30 ng) or with DNA-PKcs plus Ku and assayed for DNA binding activity by EMSA with the 40 bp probe as described in Chapter II (Materials and Methods). Further, the MonoS pool (30 and 60 ng) either untreated, treated with Centricon 100, or boiled, was incubated with DNA-PKcs and Ku to assay for the formation of complex *D*. Lastly, BSA and Ovalbumin were added at two different amounts into EMSA reaction mixtures containing DNA-PKcs and Ku to determine their effects. The contents of each lane are as follows: *Lane 1* - probe alone; *lane 2* - Ku; *lane 3* - DNA-PKcs; *lane 4* - MonoS pool; *lane 5* - Ku plus MonoS pool; *lane 6* - DNA-PKcs plus MonoS pool; and *lane 7* - DNA-PKcs plus Ku. *Lanes 8 - 20* all contained DNA-PKcs plus Ku and the following samples: *lanes 8* and *9* - 30, 60 ng of untreated MonoS pool; *lanes 10* and *11* - 30, 60 ng of MonoS sample retained in Centricon 100 microconcentrator; *lanes 12* and *13* - 2 and 4 µL of MonoS sample that passed through the Centricon 100 microconcentrator; *lanes 14* and *15* - 30, 60 ng of boiled MonoS pool; *lanes 17* and *18* - 50, 100 ng of BSA; *lanes 11* and *20* - 50, 100 ng of Ovalbumin. The putative DNA-PKcs-Ku-DNA complex is indicated by upper case *D*.

any new protein-DNA complexes when incubated with the DNA probe alone, with Ku alone, or with DNA-PKcs alone (Figure IV-3, *lanes 4, 5* and *6*, respectively). Addition of chemical cross-linkers to reactions containing the 40 bp probe with either the MonoS pool alone, MonoS pool plus Ku, or MonoS pool plus DNA-PKcs did not produce the formation of any new protein-DNA complexes, although complex *a, b* and *D* did show a slight increase in their migration position (data not shown). Taken together, these observations indicate the existence of a heat-labile factor, that is at least 100 kDa in molecular mass, within the MonoS pool that is responsible for the formation of a stabilized complex between DNA-PKcs, Ku and the 40 bp DNA probe.

In order to determine whether or not DNA-PKcs was present in complex *D*, total IgG proteins from rabbit anti-serum against DNA-PKcs (DPK1) and pre-immune serum were purified as described in Chapter II (Materials and Methods). Incubation of purified IgG from the DPK1 antiserum with DNA-PKcs, Ku, MonoS pool and the 40 bp DNA probe ablated the formation of complex *D* (Figure IV-5, *lanes 7* and *8*). The presence of radioactivity retained in the well suggests that the antibodies may have "super-shifted" the *D* complex. Alternatively, the antibodies may have prevented the formation of complex *D*. In either case, these data suggest that DNA-PKcs is required for the formation of complex *D*. Incubation of purified IgG from the pre-immune serum with DNA-PKcs, Ku, MonoS pool and the 40 bp DNA probe had no effect on the formation of complex *D* (Figure IV-5, *lanes 5* and *6*). The presence of Ku was detected in complex *a, b* and *D* by western blot analysis of EMSA gels transferred to a PVDF membrane (data not shown).

We have shown that polypeptides in the MonoS pool, which included NF90/45 and eIF-2 were able to promote the formation of a complex (complex *D*) that was similar to the stabilized complex (complex *C*) formed between DNA-PKcs and Ku on DNA in the presence of chemical cross-linkers. The presence of DNA-PKcs in complex *D* was confirmed by alteration of complex *D* by purified IgG to DNA-PKcs in EMSA assays (Figure IV-5), while the presence of Ku in complex *D* was confirmed by a Western blot (data not shown). Under our EMSA conditions, purified DNA-PKcs and Ku did not appear to interact with DNA unless chemical cross-linkers or the proteins in the MonoS pool were present.

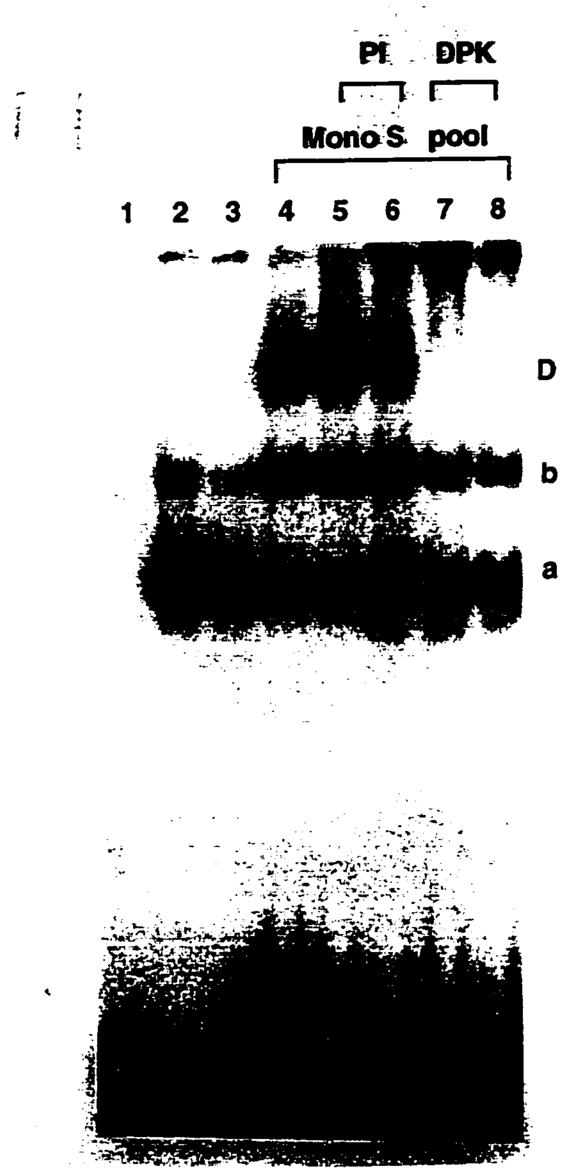

Figure IV-5: EMSA studies with purified IgG proteins from DPK-1 antiserum and pre-immune antiserum, MonoS pool, Ku, DNA-PKcs and the 40 bp DNA probe. Immunoglobulin proteins were purified from DPK-1 antiserum or pre-immune antiserum and tested for its effect on the formation of complex *D* as described in Chapter II (Materials and Methods). The contents of each lane are as follows: *lane 1* - probe alone; *lane 2* - Ku (10 ng) and *lane 3* - Ku plus DNA-PKcs (30 ng). *Lanes 4-8* contained DNA-PKcs, Ku and the MonoS pool along with the following: *lanes 5, 6* - 0.1 and 0.2 μg of IgG from pre-immune serum; *lanes 7, 8* - 0.1 and 0.2 μg of IgG from DPK1 serum.

Recently, several groups have reported the formation of DNA-PKcs-Ku complexes with DNA in EMSA studies (Yaneva *et al.*, 1997; Hammarsten and Chu, 1998). However, the complexes claimed to contain DNA-PKcs and Ku in these reports did not appear to be discrete protein-DNA complexes; rather, the radioactivity signal from the probe appeared smeared and/or retained in the well. These results further support the idea the interaction between DNA-PKcs and Ku on DNA is transient or unstable. Therefore, minor differences in assay conditions may influence the interaction of DNA-PKcs with Ku on DNA resulting in different appearances in EMSA studies. Under our EMSA conditions, chemical cross-linkers or the polypeptides present in the MonoS pool stabilized the formation of the DNA-PKcs-Ku-DNA complex, resulting in a protein-DNA complex with its radioactive probe signal appearing as a discrete band with little to no smearing or retention in the wells.

Lieber and colleagues reported that highly purified DNA-PKcs interacts with a blunt ended 18 bp DNA probe in the absence of Ku (Yaneva *et al.*, 1997). Under our EMSA conditions, we did not observe binding of DNA-PKcs to the 40 bp DNA probe either in the presence or absence of chemical cross-linkers. The difference may be attributed to the sizes of DNA used in each assay. However, we did not observe DNA-PKcs-DNA binding activity with a blunt ended 20 bp DNA probe, derived from our 40 bp DNA probe, either (data not shown). Similarly, Gottlieb and Jackson (1993) have shown by UV cross-linking, DNA-PKcs does not bind 30 bp DNA unless Ku is present. Lieber and colleagues have recently investigated the kinetics of DNA-binding activity of DNA-PKcs for a 35 bp duplex oligonucleotide using surface plasmon resonance (SPR) (West *et al.*, 1998). They reported that DNA-PKcs has extremely high association (1.5×10^{7} $M^{-1}s^{-1}$) and dissociation rates (0.048 s^{-1}) that yielded an equilibrium dissociation constant (K_d) of 3.1×10^{-9} M (West *et al.*, 1998). These high association and dissociation rates may be a plausible explanation for the differences observed in the DNA binding activity of DNA-PKcs. Since the DNA-PKcs-DNA complex is highly unstable, it is likely very sensitive to changes in equilibrium and therefore minor differences in assay conditions in EMSA studies may yield different results. Nevertheless, under our EMSA conditions, we observed that a stabilized complex between DNA-PKcs, Ku and DNA is formed only in the presence of chemical cross-linkers or proteins in the MonoS pool.

IV.3: Summary

We have shown that chemical cross-linkers or proteins present in the MonoS pool, which contained NF90/45 and eIF-2, are needed to stabilize the interaction between DNA-PKcs and Ku on DNA in EMSA studies.

CHAPTER V

RESULTS AND DISCUSSION - PART 3

INTERACTION OF NF90/45 WITH DNA-PK

Chapter V - Interaction of NF90/45 with DNA-PK[3]

V.1: Introduction

We have shown that the polypeptides present in a protein sample pooled from fractions eluted from MonoS-FPLC (called MonoS pool) can stabilize the interaction between DNA-PKcs and Ku on DNA. Silver-stained gels of the individual fractions eluted from MonoS-FPLC (Chapter III, Figure III-2) and the MonoS pool (data not shown) revealed 6 predominant polypeptides: NF90, p75, eIF-2α, 2β, 2γ and NF45. To date, we have not been successful in purifying NF90/45 away from eIF-2. Therefore, in order to determine specifically which polypeptides were responsible for the observed "stabilizing activity", recombinant NF90 and NF45 (rNF90/rNF45) and purified rat liver eIF-2 and recombinant eIF-2α were tested for their ability to stabilize the interaction between DNA-PKcs and Ku on DNA in EMSA studies.

V.2: Results and Discussion

a) EMSA studies with recombinant NF90/45 and purified eIF-2

Recombinant NF90 and rNF45 with C-terminal histidine tags were purified from bacteria and assessed for the presence of the "stabilizing activity" via EMSA studies as described in Chapter II (Materials and Methods). Incubation of rNF90 or rNF45 with the 40 bp DNA probe either together or separately did not produce any DNA-protein complexes under the conditions of our assay (Figure V-1, *lanes 4-7*). Similarly, addition of rNF90 or rNF45 and rNF90 plus rNF45 to samples containing Ku and the 40 bp DNA probe did not alter the Ku-DNA complexes *a* and *b* (Figure V-1, *lanes 8-11*). Addition of rNF90 or rNF45 and rNF90 plus rNF45 to samples containing DNA-PKcs and the 40 bp DNA probe also did not produce any new protein-DNA complexes (Figure V-1, *lanes 12-15*). However, rNF90 alone incubated with DNA-PKcs, Ku and the 40 bp DNA probe induced the formation of a protein-DNA complex that migrated at a similar position to the previously described complex *D* (labeled *D'* in Figure V-1, *lane 17*). Complex *D'* was not produced by incubation of DNA-PKcs, Ku and the 40 bp DNA

[3] Figures V-1 and V-7 presented in this chapter have been published: Ting, N.S.Y., Kao, P.N., Chan, D.W., Lintott, L.G., and Lees-Miller, S.P. (1998) *DNA-dependent Protein Kinase Interacts with Antigen Receptor Response Element Binding Proteins NF90 and NF45*. J. Biol. Chem. 273(4):2136-2145.

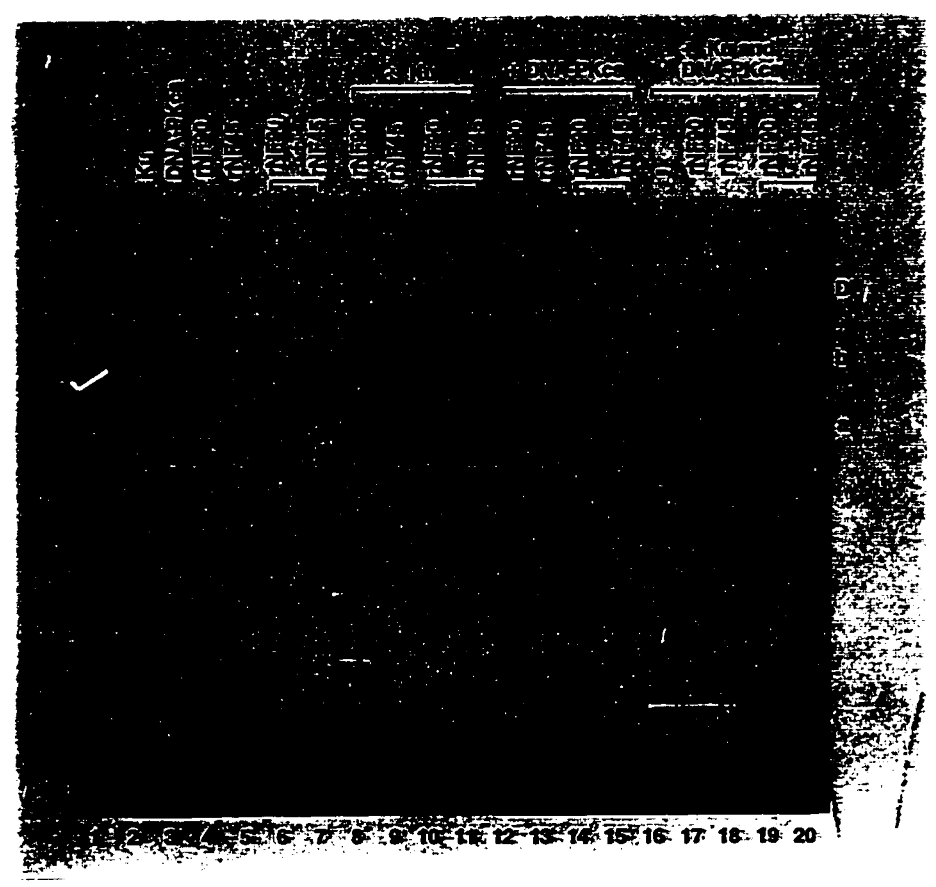

Figure V-1: EMSA studies with recombinant NF90/45, DNA-PKcs, Ku and the 40 bp DNA probe. Recombinant NF90 and NF45 (rNF90/rNF45) were purified from bacteria and assayed for their ability to stabilize the interaction between DNA-PKcs and Ku on DNA as described in Chapter II (Materials and Methods). The contents of each lane are as follows: *lane 1* - free probe; *lane 2* - Ku (10 ng) ; *lane 3* - DNA-PKcs (30 ng); *lane 4* - rNF90 (200 ng); *lane 5* - rNF45 (200 ng); *lanes 6,7* - rNF90 plus rNF45 (100 ng and 200 ng each, respectively). *Lanes 8-11* contained Ku and the following: *lane 8* - rNF90; *lane 9* - rNF45; *lanes 10,11* - rNF90 plus rNF45 as before. *Lanes 12-15* contained DNA-PKcs and the following: *lane 12* - rNF90; *lane 13* - rNF45, *lanes 14,15* - rNF90 plus rNF45. *Lanes 16-20* contained DNA-PKcs and Ku with the following: *lane 16* - no other proteins; *lane 17* - rNF90; *lane 18* - rNF45; *lanes 19, 20* - rNF90 plus rNF45. The Ku-DNA complexes are indicated as *a* and *b*, and the protein-DNA complex similar to the previously described complex *D* is indicated by *D'*.

probe with rNF45 alone (Figure V-1, *lane 18*), but was formed when rNF90 plus rNF45 were incubated with DNA-PK and the 40 bp DNA probe (Figure V-1, *lanes 19* and *20*). Consistent with previous results, addition of chemical cross-linkers into these reactions slightly increased the mobility of the existing protein-DNA complexes but did not promote the formation of any new protein-DNA complexes (data not shown). These data suggest that rNF90 alone or rNF90 plus rNF45 is able to stabilize the interaction between DNA-PKcs, Ku on DNA. Furthermore, it implicates NF90 as the factor that may be responsible for the "stabilizing activity" present in the MonoS pool of polypeptides.

In order to determine whether or not eIF-2 could also stabilize the interaction of DNA-PKcs and Ku on DNA, purified rat liver eIF-2 and recombinant eIF-2α were obtained from Dr. Scott Kimball (Pennsylvania State University) and tested in our EMSA studies. Western blot analysis of the rat liver eIF-2 and recombinant eIF-2 samples with antibodies against rNF90 and rNF45 showed the absence of any cross-reacting polypeptides (data not shown). Incubation of rat liver eIF-2 and recombinant eIF-2α with the 40 bp DNA probe displayed no DNA binding activity (Figure V-2, *lanes 3* and *4*). Addition of rat liver eIF-2 and recombinant eIF-2α to Ku or DNA-PKcs alone and Ku plus DNA-PKcs with the 40 bp DNA probe resulted in no formation of any new protein-DNA complexes (Figure V-2, *lanes 5-13*). These data show that rat liver eIF-2 and recombinant eIF-2α do not stabilize the interaction between DNA-PKcs and Ku on DNA, suggesting that eIF-2 does not contribute to the "stabilizing activity" seen in the MonoS pool. However, we note that it is possible that human eIF-2 may have different properties than eIF-2 purified from rat liver. To date, we have been unable to obtain recombinant or purified human eIF-2.

b) Further EMSA studies with rNF90

Recombinant His-tagged NF90 used in the EMSA studies was purified over a single Ni-chelating column. Coomassie Blue stained SDS-PAGE gels of rNF90 samples revealed that rNF90 was present at about 90% purity (data not shown). Further attempts to purify this polypeptide using FPLC-MonoQ or phosphocellulose resins resulted in very poor yields of protein. Although extreme caution was taken during the purification procedure, rNF90 was extremely unstable and easily proteolysed. Similar behavior has

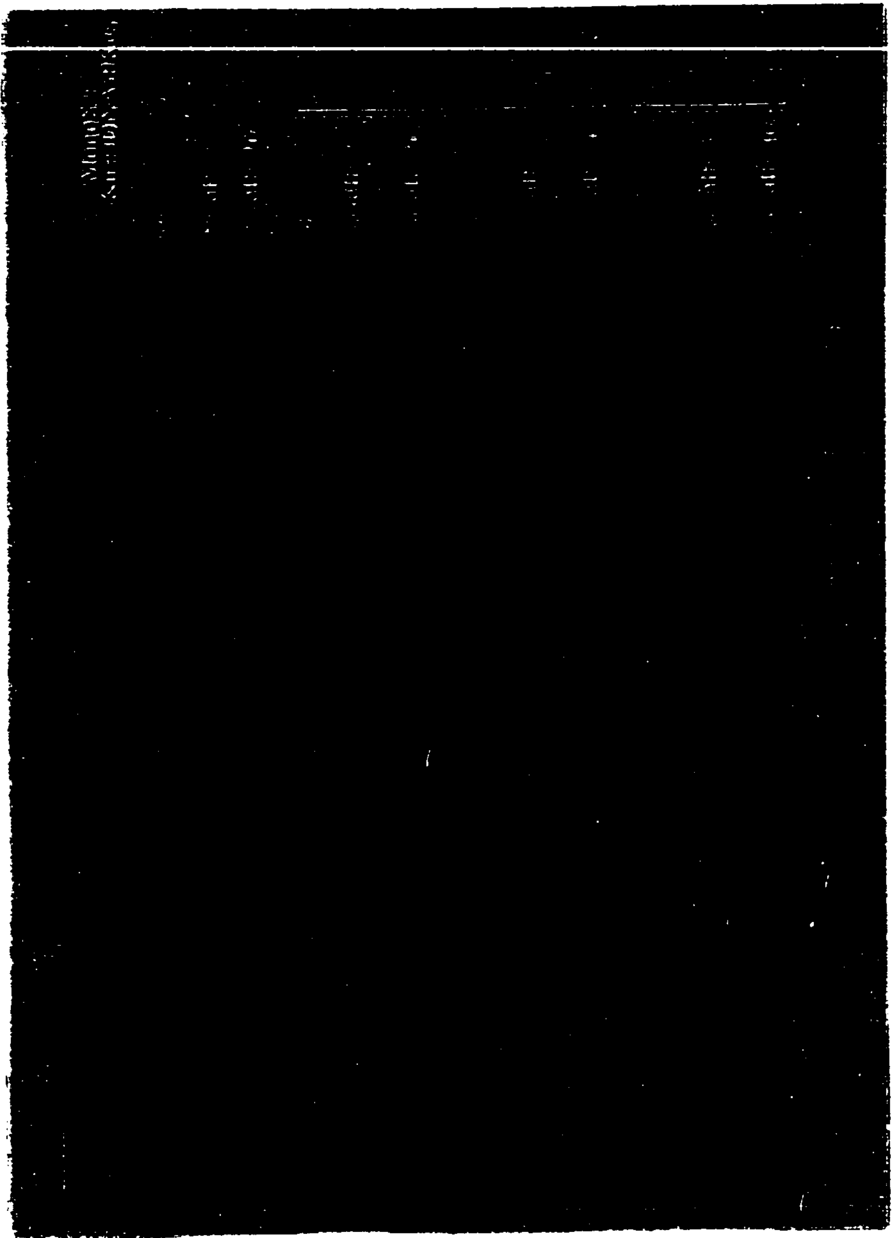

Figure V-2: EMSA studies with Rat liver eIF-2 and recombinant eIF-2α, DNA-PKcs, Ku and the 40 bp DNA probe. Rat liver eIF-2 and recombinant eIF-2α were kind gifts from Dr. Scott Kimball (Pennsylvania State University). These samples were incubated with DNA-PKcs, Ku and 40 bp probe to determine their affects on DNA-PK-DNA interactions as described in Chapter II (Materials and Methods). The contents of each lane are as follows: *lane 1* - Ku (10 ng), DNA-PKcs (30 ng) plus MonoS pool (30 ng); *lane 2* - free 40 bp probe; *lane 3* - rat liver eIF-2 (100 ng); *lane 4*- recombinant eIF-2α (100 ng). *Lanes 5-7* contained Ku with the following: *lane 5*- Ku alone; *lane 6*- eIF-2; *lane 7* - eIF-2α. *Lanes 8-10* contained DNA-PKcs with the following: *lane 8* - DNA-PKcs alone; *lane 9* - eIF-2, *lane 10* - eIF-2α. *Lanes 11-13* contained DNA-PKcs and Ku with the following: *lane 11*- no other proteins; *lane 12*- eIF-2; *lane 13*- eIF-2α. The stabilized DNA-PKcs/Ku/DNA complex in the presence of the MonoS pool is indicated by upper case *D* as before.

been noted by Dr. Peter Kao (personal communications). This unstable nature of rNF90 could be attributed to the fact that rNF90 was present in bacterial inclusion bodies and urea was used to solubilize the polypeptide. Therefore, a series of control experiments was performed to ensure that rNF90, and not a contaminating bacterial protein, was responsible for the "stabilizing" activity. First, rNF90 was concomitantly purified from bacterial extracts that were induced or not induced for rNF90 expression and assayed for the stabilizing activity with DNA-PKcs, Ku and the 40 bp DNA probe. The formation of the *D'* complex was promoted only in the presence of rNF90 purified from extracts that were induced for rNF90 expression but not in uninduced bacterial extracts (compare *lane 4* to *lane 5* in Figure V-3).

Second, an immunodepletion experiment was performed in which rNF90 samples were initially incubated with protein G-Sepharose beads that were either untreated or had been pre-coupled with antibodies from mouse pre-immune or anti-NF90 serum (see Chapter II for details). Following incubation with the protein G-Sepharose beads, the supernatants were recovered and either added to DNA-PKcs, Ku and the 40 bp DNA probe to assay for the "stabilizing activity", or analyzed for their content by silver staining of SDS gels (Figure V-4). The supernatants recovered from samples of rNF90 incubated with beads alone or beads precoupled with pre-immune antibodies were able to induce the formation of the *D'* complex in the presence of Ku, DNA-PKcs and the 40 bp DNA probe (seen in *lanes 5* and *6*, Figure V-4B). The presence of rNF90 in these samples was confirmed by the silver-stained SDS gel (*lanes ii and iii*, Figure V-4A). On the other hand, supernatant from samples in which rNF90 was incubated with beads pre-coupled with antibodies against NF90 failed to promote the formation of complex *D'* (*lane 7*, Figure V-4B). Analysis of the supernatant by SDS-PAGE followed by silver staining, revealed that rNF90 has been immunodepleted (*lane iv*, Figure V-4A). Taken together, these data strongly suggest that rNF90 is required for the stabilization of DNA-PKcs, Ku on DNA in our EMSA studies.

c) EMSA studies with rNF90 polypeptides containing deletions

In EMSA experiments in which individual aliquots from fractions eluting from the MonoS-FPLC column were tested for the ability to stabilize the interaction between

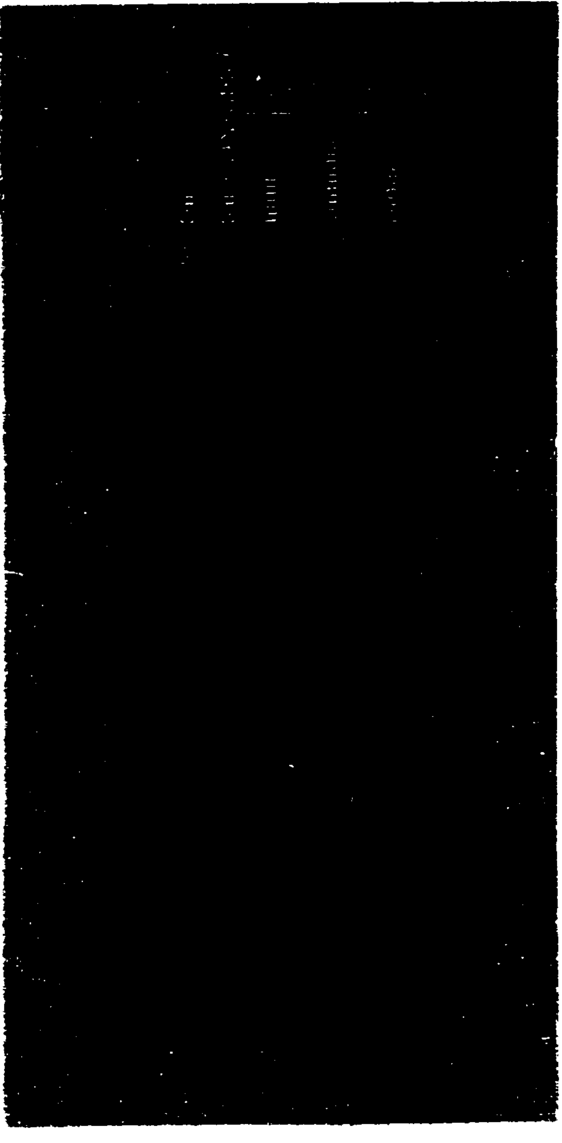

Figure V-3: EMSA studies with rNF90 purified from extracts from bacteria induced and uninduced for the expression of NF90. rNF90 was purified from bacterial extracts that were induced or not induced for the expression of rNF90 and assayed for its ability to form a stabilized complex between DNA-PKcs and Ku on DNA as described in Chapter II (Materials and Methods). The contents of each lane are as follows: *lane 1* - free probe; *lane 2* - Ku (10 ng); *lane 3* - Ku plus DNA-PKcs (30 ng). *Lanes 4-6* contained DNA-PKcs and Ku with the following: *lane 4* - 100 ng of rNF90 purified from induced bacteria extracts; *lane 5* - 100 ng of total protein purified from uninduced bacteria extracts; *lane 6* - MonoS pool (30 ng). The stabilized DNA-PKcs-Ku-DNA complex is indicated by *D'*.

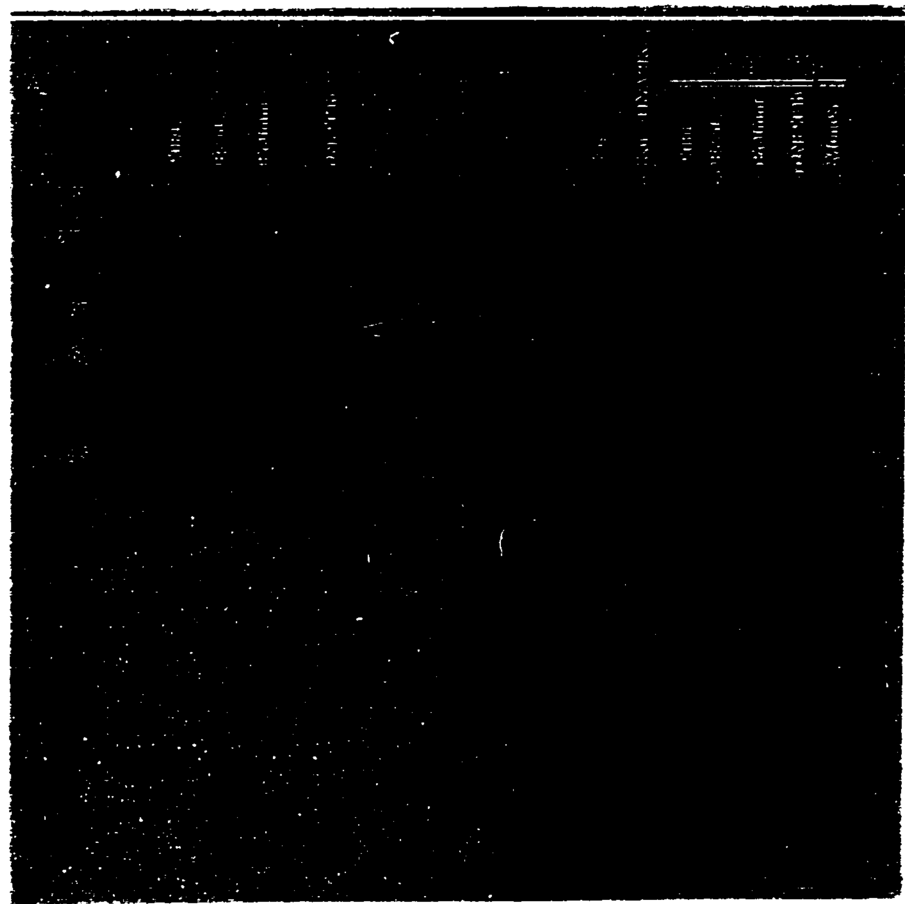

Figure V-4: Immunodepletion experiment with rNF90. Approximately 0.5 μg of rNF90 (10 μL) samples were initially incubated with protein G-Sepharose beads alone or precoupled with antibodies from mouse pre-immune sera and NF90 anti-sera. Supernatants were removed following incubation and its contents were equally divided to be analyzed by SDS-PAGE (10% acrylamide) or tested in EMSA studies as described in Chapter II (Materials and Methods).

A) Silver-stained SDS gel of Immunodepleted rNF90 samples. 5 μL of rNF90 or immunodepleted samples were fractionated on SDS-PAGE and silver stained. The contents of each lane are as follows: *lane i* - rNF90; *lane ii* - rNF90 incubated with beads alone; *lane iii* - rNF90 incubated with beads precoupled with pre-immune serum; *lane iv* - rNF90 incubated with beads pre-coupled with anti-NF90 serum. The polypeptide corresponding to rNF90 is indicated by the arrow.

B) EMSA studies with Immunodepleted rNF90 samples. 5 μL rNF90 (0.25 μg) or the immunodepleted rNF90 samples were added to EMSA reactions containing DNA-PKcs, Ku and the 40 bp DNA probe and the products analyzed by non-denaturing gel electrophoresis and autoradiography. The contents of each lane are as follows: *lane 1* - free probe; *lane 2* - Ku (10 ng); *lane 3* - Ku plus DNA-PKcs (30 ng). *Lanes 4 - 8* contained Ku plus DNA-PKcs and the following: *lane 4* - rNF90; *lane 5* - rNF90 incubated with beads alone; *lane 6* - rNF90 incubated with beads precoupled with pre-immune antibodies; *lane 7* - rNF90 incubated beads pre-coupled with antibodies against NF90; *lane 8* - MonoS pool (30 ng). The presence of the stabilized DNA-PKcs-Ku-DNA complex is indicated by *D'*.

DNA-PK and DNA, fractions which contained predominantly p75 and NF45 (Figure III-2, *lanes 44,46*) were able to induce the formation of complex *D* (Figure IV-2, *lane 13)*. In light of the evidence that suggests that NF90 alone is required for stabilizing the interaction between DNA-PKcs and Ku on DNA, this observation was intriguing. Protein fractions which contained p75, which we speculated to be either a proteolytic product or a variant form of NF90, retained the "stabilizing activity". This suggested to us that although p75 may be different from NF90, it contained amino acid sequences that are required for the formation of complex *D*. We therefore sought to identify these amino acid sequences by making deletion fragments of rNF90 and assaying for their ability to stabilize the interaction of DNA-PKcs and Ku on DNA.

Unique restriction sites within the cDNA sequence of NF90 were utilized to make five plasmid constructs expressing rNF90 deletion fragments as described in Chapter II (Table II-3; Materials and Methods) (Figure V-5). All five of the rNF90 constructs were expressed in bacteria; however, each recombinant NF90 protein was present as an insoluble protein in inclusion bodies. Therefore the recombinant NF90 proteins containing deletions were purified by the same methods employed to purify rNF90 and rNF45. These rNF90 deletion fragments were also found to be extremely labile and attempts to further purify them resulted in very little success. Nevertheless the samples obtained from the Ni-chelating column were approximately 90% pure as assessed by Coomassie Blue stained gels. The amount of rNF90 deletion polypeptides present was quantitated by initially scanning the Coomassie Blue stained gel and subjecting the image to densitometric analysis as described in Chapter II (Materials and Methods). The rNF90 deletion fragment samples were normalized to give equal densitometric units per µL, prior to use in EMSA reactions. Individual incubation of the five rNF90 deletion fragments with the 40 bp DNA probe resulted in no formation of any protein-DNA complexes (*lanes 4-8*, Figure V-6). Consistent with previous results, rNF90FL, rNF45 and the MonoS pool did not show any DNA binding activity (*lanes 2,3* and *9*, Figure V-6). In the presence of DNA-PKcs, Ku and the 40 bp DNA probe, full length rNF90 (90FL) and the MonoS pool induced the formation of complex *D'* (*lanes 12* and *19*, Figure V-6). Deletion fragments designated as rNF90D and rNF90PH were able to induce the formation of *D'* complexes that were similar in intensity to that seen with full

rNF90 Deletion Fragment	Amino Acids	DNA-PKcs/Ku/DNA Stabilizing Activity
rNF90FL	1 - 671	+++
rNF90D (HindIII)	1 - 591	+++
rNF90RV (EcoRV)	1 - 314	-
rNF90H3 (EcoRV-HindIII)	314 - 591	++
rNF90SP (Sal1-Pst1)	152 - 417	+
rNF90PH (Pst1-HindIII)	417 - 591	+++

1 100 200 300 400 500 600 700

Amino Acids

Figure V-5: Deletion fragments of rNF90 and their ability to stabilize the interaction between DNA-PKcs and Ku.[4] pQE constructs expressing various deletion products for rNF90 were created by using restriction enzyme digest of specific sites within the NF90 cDNA sequence as described in Chapter II (Materials and Methods). In the first column, the names assigned to each rNF90 deletion fragment along with the restriction enzymes used (in brackets) to create the expression construct are listed. The amino acid composition of each deletion fragment is indicated in the second column. Indicated in the third column is the following arbitrary scale to score the ability of the polypeptide to stabilize the interaction between DNA-PKcs and Ku on DNA: +++ = Similar in intensity compared to wild type rNF90FL; ++ = less intense and, + = very weak compared to wild type NF90 (full length); - = indicates no stabilization observed.

[4] The expression vectors for the NF90 deletion fragments were prepared by Ruiqiong Ye.

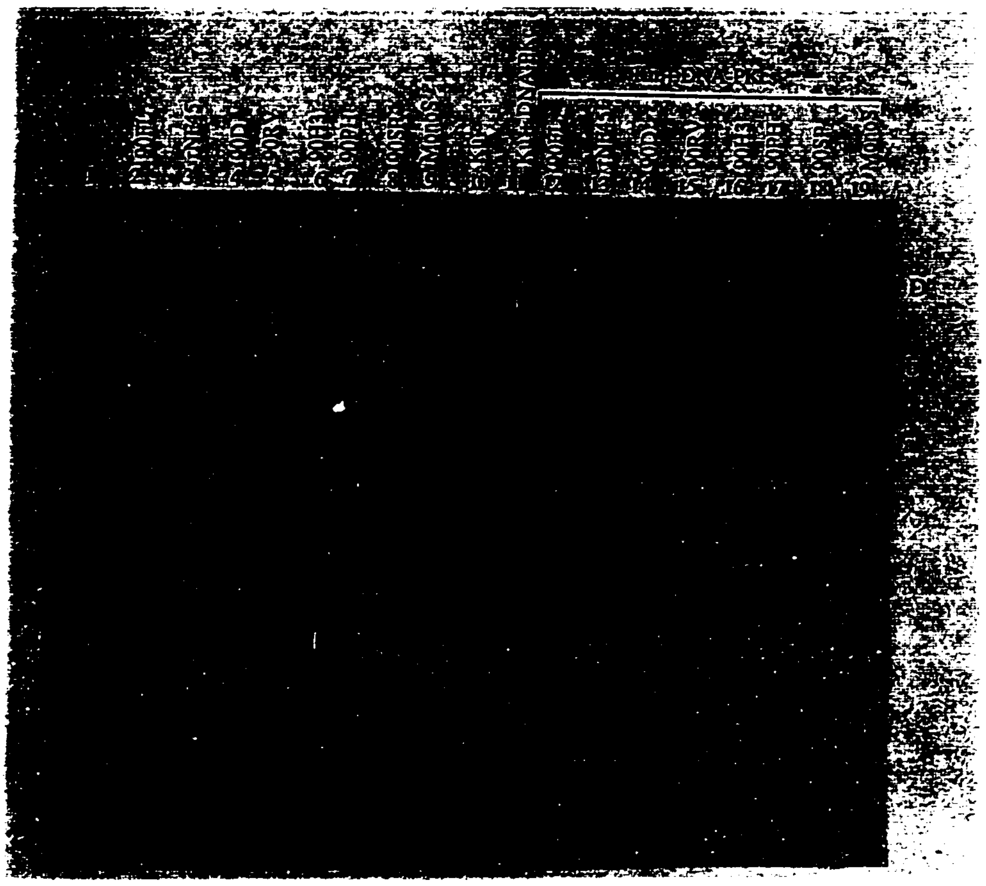

Figure V-6: EMSA studies with deletion rNF90 fragments, DNA-PKcs, Ku and the 40 bp DNA probe. The rNF90 polypeptides were purified and quantitated according to Chapter II (Materials and Methods). Among the recombinant protein preparations, the rNF90D sample was considered to be the most homogeneous as judged by Coomassie Blue-stained gels. Therefore all of the recombinant proteins were diluted based on the assigned arbitrary densitometric units to obtained approximately 0.2 µg of rNF90D. The diluted recombinant protein samples were added to EMSA reactions containing either probe alone or with DNA-PKcs, Ku and probe to assess their effects. The contents of each lane are as follows: *lane 1* - free probe; *lanes 2-9* contained recombinant proteins or the MonoS pool as indicated; *lane 10* - Ku (10 ng); *lane 11* - Ku plus DNA-PKcs (30 ng). *Lanes 12-19* contained Ku plus DNA-PKcs and the recombinant proteins or the MonoS pool as indicated. The stabilized DNA-PKcs-Ku-DNA complex is indicated by *D'*.

length rNF90 (compare *lanes 14* and *17* to *lane 12*, Figure V-7). On the other hand, the *D'* complex induced by rNF90H3 was not as intense compared to rNF90PH or rNF90D (compare *lane 16* with *lanes 17* and *14*, Figure V-6), while rNF90SP only induced a slight formation of complex *D'* (*lane 18*, Figure V-6). rNF90RV, which constituted the first 314 N-terminal amino acids of NF90, had no effect on the interaction between DNA-PK and DNA (*lane 15*, Figure V-6). The results of the EMSA studies with the deletion rNF90 fragments are summarized in Figure V-5.

It is interesting to note that rNF90H3 (amino acids 314-591) supported the formation of a stabilized DNA-PKcs/Ku/DNA complex (*D'*) of less intensity compared to the *D'* complex produced by an equal amount of rNF90PH (amino acids 417-591). This suggests that the 103 amino acids in the N-terminal half of rNF90H3 may possess an intramolecular inhibitory function; elimination of these amino acids relieved the potential inhibitory effect, yielding a polypeptide (rNF90PH) that was more capable of stabilizing the interaction of DNA-PKcs and Ku on DNA. These potential "inhibitory amino acid sequences" between amino acids 314-417 could possibly account for the much poorer stabilizing activity of rNF90SP which contained amino acids 152-417 (Figure V-5). Alternatively, these polypeptides may not have folded properly to yield a stable protein, since urea was used to solubilize these polypeptides during the purification procedure. Therefore, caution must be taken in the interpretation of the results from this experiment. Nevertheless, these data showed that, rNF90PH, which contains amino acids 417-591 of NF90, represented a region with the minimum number of amino acids that was able to stabilize the interaction of DNA-PKcs and Ku on DNA to an extent comparable with full length rNF90.

d) Antibody immunoprecipitation studies.

To further investigate the possible interaction between DNA-PK and NF90/45, mouse polyclonal antibodies to rNF90, rNF45 and monoclonal antibodies against DNA-PKcs were used to immunoprecipitate polypeptides from a partially purified placenta protein fraction containing DNA-PK and proteins in the MonoS pool (pre-dsDNA-cellulose). Monoclonal antibodies to SV40 T-Ag and mouse pre-immune sera were used as controls. Antibodies were incubated with the pre-dsDNA fraction under EMSA assay

conditions in the presence or absence of the 40 bp DNA and immunoprecipitations were carried out as described in Chapter II (Materials and Methods). Precipitated immune complexes were analyzed by Western immunoblot using rabbit polyclonal antibodies to NF90 and DNA-PKcs (DPK1) (Figure V-7). DNA-PKcs was immunoprecipitated with NF90 and NF45 with antibodies against rNF90 and rNF45, either in the presence or absence of DNA (*lanes 1,2,3* and *4*, Figure V-7, and data not shown). Ku was not detected in these immune complexes, suggesting that NF90 and NF45 can interact with DNA-PKcs in the absence of Ku and DNA. It is interesting to note that NF90 and NF45 were not precipitated with the monoclonal antibodies to DNA-PKcs (42-27) (Figure V-7, *lanes 5* and *6*). These antibodies, which were made to a 150 kDa region in the C-terminus of DNA-PKcs, may have disrupted the interaction between DNA-PKcs and NF90/45 (Song *et al.*, 1996). Alternatively, the interaction between NF90/45 and DNA-PKcs may have masked the epitope site recognized by the DNA-PKcs monoclonal antibodies. DNA-PKcs, NF90 and NF45 were not precipitated by the control SV40 T Ag monoclonal antibody (*lane 7*, Figure V-7) or pre-immune serum (*lane 8*, Figure V-7). Antibodies to NF90 and NF45 immunoprecipitated p90 and p75, lending further support to the notion that p75 is related to NF90. These data also support the notion that NF90 and NF45 exist as a heterodimer. Since an ammonium sulfate percipitation step was used to obtain the partially purified protein fraction used in this immunoprecipitation experiment, the interaction between DNA-PKcs and NF90/45 may be an *in vitro* artifact arising from the treatment of proteins with ammonium sulfate. We therefore performed similar experiments using crude extracts from Jurkat T-cells, which were not treated with ammonium sulfate. As seen in Figure V-8 (and data not shown), DNA-PKcs, NF90 and NF45 co-immunoprecipitated from crude Jurkat T-cell extracts using antibodies to rNF45. However, Ku was not detected in these immune complexes (data not shown). Collectively, these data suggest that DNA-PK may interact with NF90/45 *in vivo*, via an interaction between DNA-PKcs and NF90.

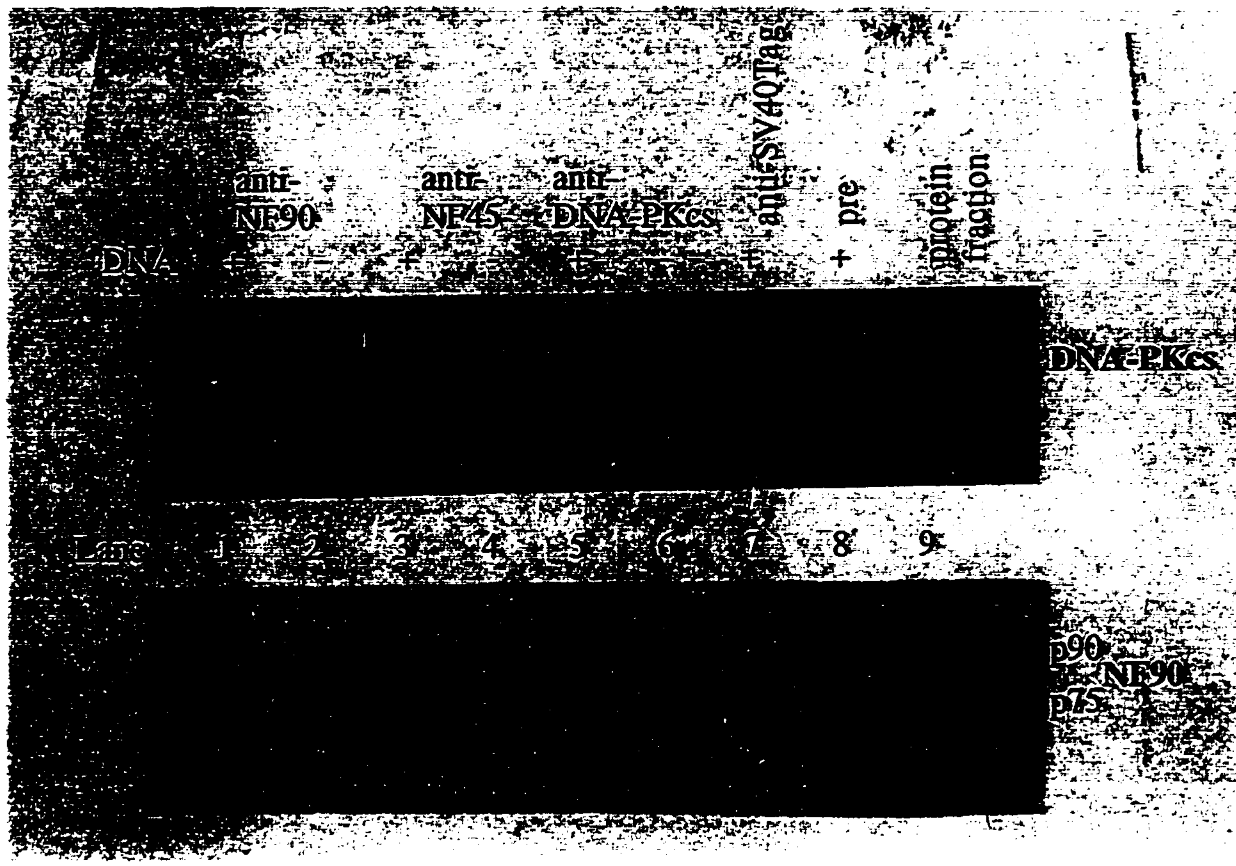

Figure V-7: Immunoprecipitation studies with partially purified protein fraction from human placenta. Approximately 20 μg of a fraction from the pre-dsDNA-cellulose chromatography purification step was incubated under EMSA conditions in the presence or absence of 5 μg/mL of the 40 bp DNA as indicated. The following antibodies were added to the protein fraction for immunoprecipitation: *lanes 1* and *2* - mouse polyclonal antibodies rNF90; *lanes 3* and *4* - mouse polyclonal antibodies to rNF45; *lanes 5* and *6* - monoclonal antibodies to DNA-PKcs (42-27); *lane 7* - control monoclonal antibodies to SV40 T-Ag and *lane 8* - pre-immune sera. *Lane 9* contained approximately 5 μg of the pre-dsDNA-cellulose fraction. Western blot analysis of the resulting protein immune-complexes were carried out with rabbit polyclonal antibodies to DNA-PKcs (top panel) and NF90 (bottom panel). Antibodies to NF90 and NF45 immunoprecipitated both p90 and p75.

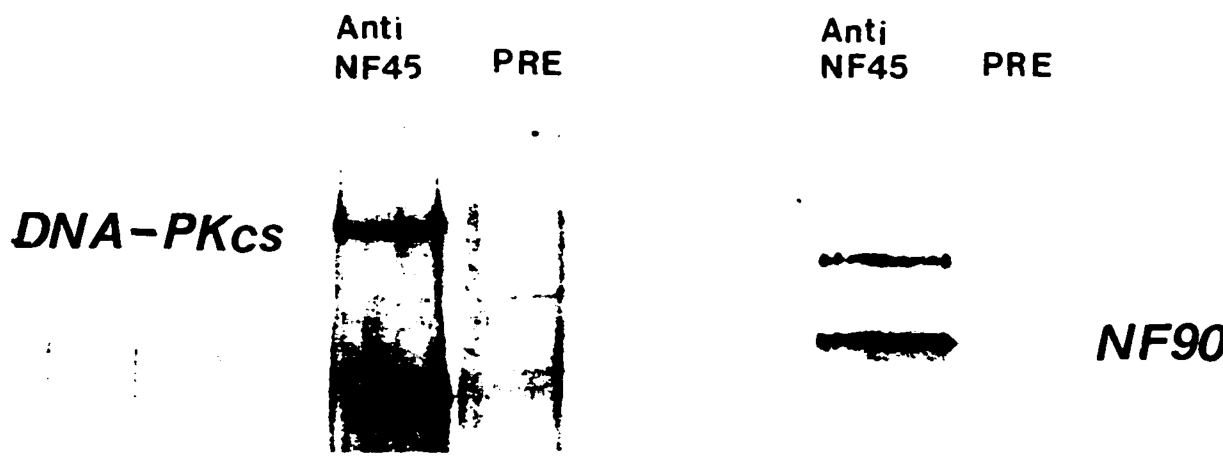

Figure V-8: Immunoprecipitation studies with Jurkat T-Cell crude cell extract. Mouse polyclonal antibodies to rNF45 or pre-immune serum were added to approximately 20 μg of total proteins from Jurkat T-cell crude cell extract that were pre-incubated under EMSA conditions without added DNA and immunoprecipitation was carried out as described in Chapter II (Materials and Methods). The resulting protein immune complex was analyzed by western blot with rabbit polyclonal antibodies to DNA-PKcs (DPK1) and NF90 as indicated.

e) Further Discussion

Despite the fact that NF90 and NF45 co-immunoprecipitated and were purified as a heterodimer, EMSA studies using recombinant proteins suggest that only NF90 is required for the stabilization of DNA-PKcs and Ku on DNA. Immunoprecipitation studies show that the interaction between DNA-PK and NF90/45, may be mediated solely through an interaction between DNA-PKcs and NF90, independent of Ku or DNA. Combining these studies, we propose the following model to explain the observed stabilization between DNA-PKcs and Ku on DNA as seen in EMSA. We propose that NF90 interacts initially with DNA-PKcs to form a trimeric complex of NF90/45 and DNA-PKcs. The trimeric complex, in turn, associates with Ku bound DNA or DNA bound Ku, to form the stabilized DNA-PKcs-Ku-DNA complex. Whether or not NF90 or NF45 remains associated with this stabilized complex remains to be determined. It is possible that NF90 allosterically alters the protein conformation of DNA-PKcs enabling a "tighter" association with the Ku-DNA complex. Recent atomic force microscopy (AFM) data from Lieber and colleagues suggested that DNA-PKcs may take up a position adjacent to Ku bound DNA, irrespective of any contact between the two proteins (Yaneva *et al.*, 1997). Thus, it is also possible that the trimeric DNA-PKcs/NF90/45 complex localizes to a position adjacent to Ku on the 40 bp DNA duplex, without any contact between DNA-PKcs and Ku.

Results from our EMSA studies with rNF90 deletion fragments suggest that the region spanning amino acids 417 to 591 in NF90 may be responsible for the interaction between DNA-PKcs and NF90 that resulted in the formation of stabilized complex *D'* (see Figure X-2 in Appendix, for full amino acid sequence). This amino acid region partially encompasses two regions, amino acids 419-464 and amino acids 535-604 of NF90 that were previously noted to bear some homology to dsRNA binding proteins (Kao *et al.*, 1994). These proteins include *Drosophila* maternal effect protein Staufen (STUDROME), human double-stranded RNA-activated protein kinase (PKR) and human TAR RNA-binding protein (HUMTRBP-1) (Kao *et al.*, 1994). The significance of this amino acid similarity in NF90 has yet to be explored.

V.3: Summary

We have shown that recombinant NF90 alone is able to stabilize the interaction between DNA-PKcs and Ku on DNA in EMSA studies. Moreover, we have localized the region in rNF90 that is capable of this "stabilizing activity" to between amino acids 417 and 591. Antibodies to rNF90 and rNF45 co-immunoprecipitated DNA-PKcs, NF90 and NF45 from a partially purified protein fraction and from crude cell extracts. Taken together, these data provide strong evidence that NF90/45 interacts with DNA-PKcs.

CHAPTER VI

RESULTS AND DISCUSSION - PART 4

MONO-S POOL OF PROTEINS CONTAINS HISTONE ACETYLTRANSFERASE ACTIVITY - IS NF90 A HISTONE ACETYLTRANSFERASE?

Chapter VI - MonoS pool of proteins contains Histone Acetyltransferase activity - Is NF90 a Histone Acetyltransferase?

VI.1: Introduction

NF90 and NF45 were initially discovered due to their ability to associate with the antigen receptor response element (ARRE) sequence in the promoter region of the IL-2 gene when Jurkat T-cells were treated with ionomycin and PMA. This association was inhibited by the immunosuppressent drug cyclosporin A. In *in vitro* transcription assays using nuclear extracts from stimulated Jurkat T-cells, antibodies to NF90 and NF45 inhibited transcription activity from the IL-2 promoter region. Therefore, NF90 and NF45 were initially proposed to form a heterodimeric transcription factor that was required for IL-2 expression in activated T-cells. DNA-PK has been reported to associate with a number of transcription factors including HSF1 and Oct-1 (Peterson *et al.*, 1995a; Griffin *et al.*, 1997). In the case of HSF1, this association leads to increased activity of DNA-PK towards CTD of RNA polymerase II. The presence of NF90/45 (MonoS pool) or rNF90/45 had no effect on DNA-PK activity in the SQE peptide assay (data not shown).

The amino acid sequence of NF45 shows some similarity to human DNA topoisomerase II, while NF90 has amino acid similarity to RNA binding proteins; however, to date neither activity has been found for these proteins (Kao *et al.*, 1994) (for full amino acid sequence of NF90/45, please see Figure X-2, Appendix). Upon closer examination of the amino acid sequence of NF90, there is a region between amino acids 255 and 278 that bears similarity to a conserved domain found in all histone acetyltransferases (Figure VI-1) (Drs. P.N. Kao and C.D. Allis, personal communications). Histone acetyltransferases (HAT) catalyze the transfer of an acetate moiety from acetyl-CoA to the ε-amino group of Lys residues on the amino terminal tails of histone proteins. Addition of acetate groups is believed to neutralize the positive charges on histone tails, allowing alteration of histone-DNA, histone-histone, histone-protein interactions (reviewed in Kuo and Allis, 1998). These alterations consequently remodel the chromatin, permitting better access of transcription, replication and, possibly, DNA repair proteins to the DNA. HATs have also been shown to acetylate non-histone substrates; for example, acetylation of p53 by p300/CBP HAT, stimulated p53 DNA

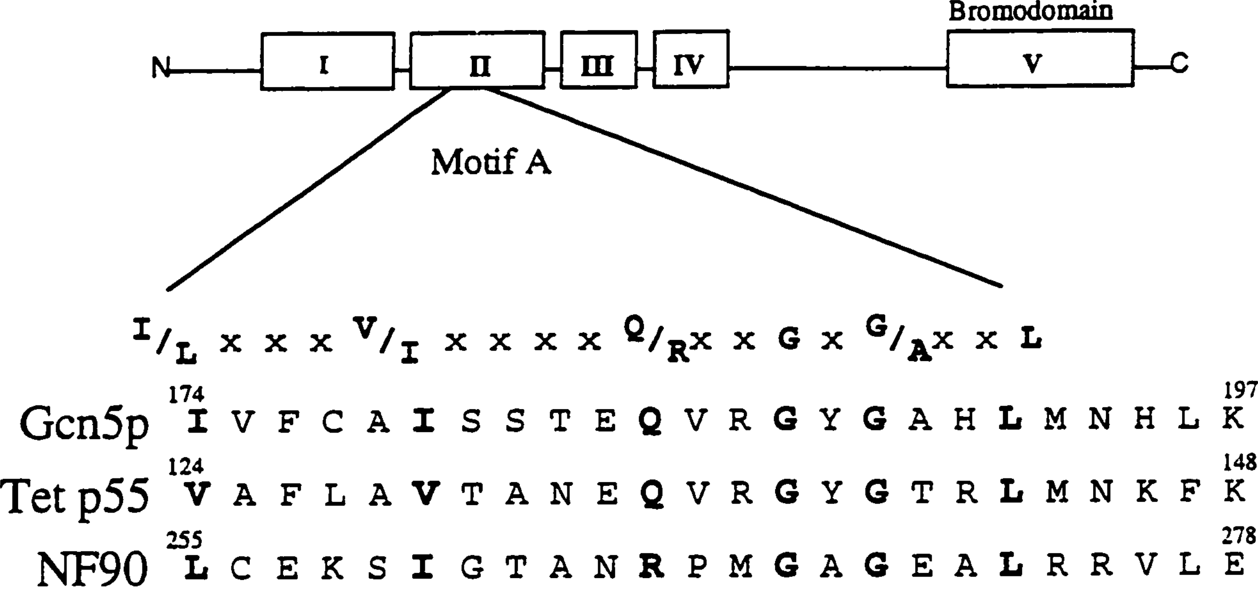

Figure VI-1: Amino acids 255 to 278 of NF90 resemble motif A of histone acetyltransferase proteins - *S. cerevisiae* Gcn5p, *Tetrahymena* p55. There are five domains (I-IV) that are conserved in all of histone acetyltransferase proteins identified to date (Neuwald and Landsman, 1997). Amino acids 255 to 278 of NF90 were aligned with the two canonical histone acetyltransferase proteins *S. cerevisiae* Gcn5p and *Tetrahymena* p55. Amino acid sequences are represented by single letter codes. The most conserved residues within motif A, are indicated as bold letters.

binding activity to its cognate DNA sequence (Gu and Roeder, 1997).

Although NF90 shows similarity in only 6 amino acids over a region of 23 amino acids, the similarity is in a region that falls within motif A in domain II of histone acetyltransferase. Motif A is the most highly conserved region of amino acids and is present in all of the HATs identified to date (Neuwald and Landsman, 1997). The amino acid sequences GxG have been proposed to be crucial for substrate binding and possibly catalysis (Dr. C.D. Allis, personal communications; reviewed in Brownell and Allis, 1996). We therefore investigated whether or not NF90 possesses HAT activity.

IV.2: Results and Discussion

0.2 μg of the MonoS pool of polypeptides that contained NF90, was tested for HAT activity by incubation with total calf thymus (CT) histones and ^{3}H-acetyl CoA under the conditions specified in Chapter II (Materials and Methods). The reaction mixtures were either spotted onto phosphocellulose filters or subjected to analysis by SDS-PAGE, fluorography and autoradiography. Phosphocellulose filters were washed in sodium carbonate and the amount of ^{3}H-acetate incorporated into the histones was quantitated by scintillation counting. Proteins in the MonoS pool supported incorporation of ^{3}H-acetate into histones but not into BSA (compare *MonoS + BSA* with *MonoS + Histones*, Figure VI-2A). The MonoS pool supported acetylation of histones H3, H4 and H2A/B (*lane 4*, Figure VI-2B). Histones H2A and H2B were not resolved in our SDS-PAGE system, therefore it is not possible to determine at this time which of these histones is acetylated. ^{3}H-acetate incorporation was not detected with reaction mixtures containing BSA and CT histones, BSA alone or with the MonoS pool with BSA (*lanes 1*, *2* and *3*, Figure VI-2B and as indicated in Figure VI-2A).

To further test the HAT activity of the MonoS pool, equal amounts (2 μg each) of total CT histones, Hela cell core histones or Hela cell mononucleosome preparation (kind gift from Dr. C.D. Allis) were used as substrates. The MonoS pool acetylated all of the histones in the total calf thymus histones, but preferentially acetylated H3 and H4 of core histones from Hela cells, and H3 and H2A/B of Hela mononucleosomes (*lanes 1, 2* and *3*, respectively; Figure VI-3). No incorporation of ^{3}H-acetate was seen with any other polypeptides in the MonoS pool or with purified DNA-PKcs and Ku (data not shown).

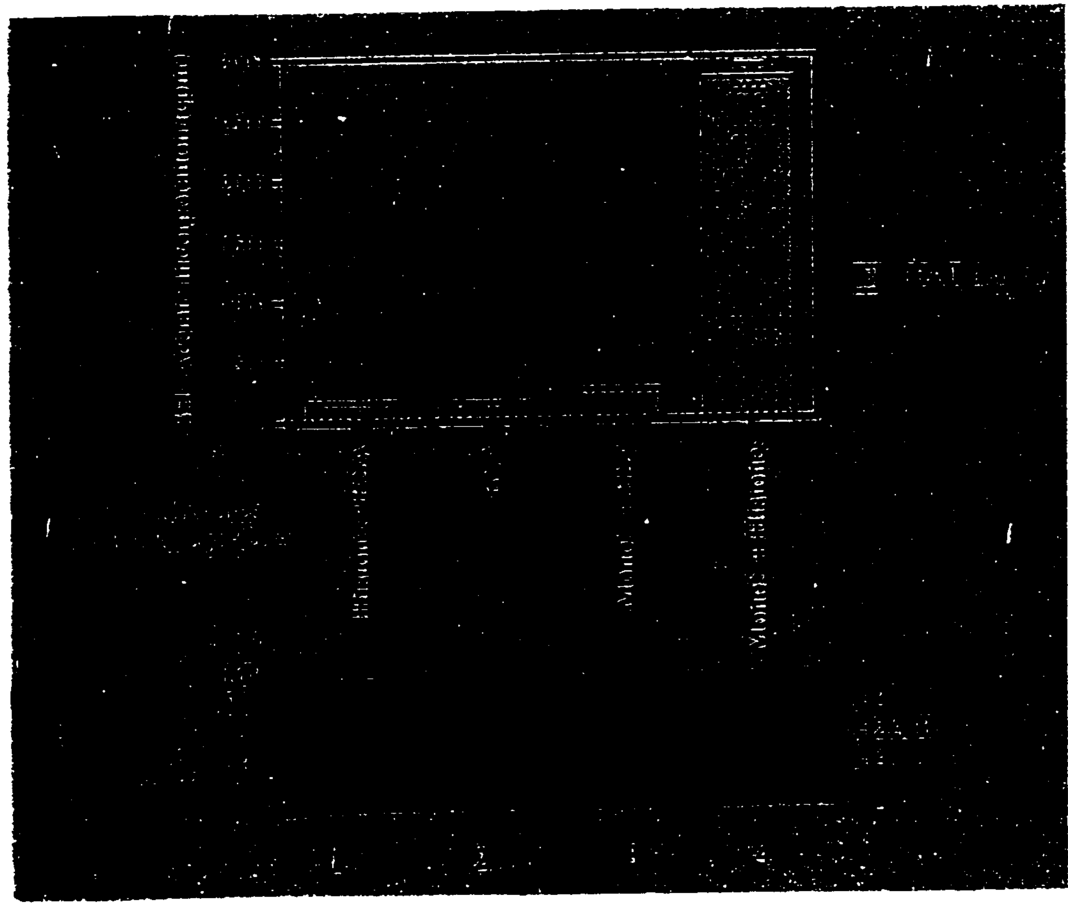

Figure VI-2: Histone acetyltransferase activity assay of the MonoS pool - A) Filter binding assays and B) SDS-PAGE analysis. Various protein samples were incubated with ^{3}H-acetyl CoA under HAT assay conditions as outlined in Chapter II (Materials and Methods). Each reaction mixture was spotted on phosphocellulose filters or fractionated on SDS-PAGE for autoradiographic analysis. The amount of ^{3}H incorporation into histones on phosphocellulose filters were quantified by scintillation counting and expressed in c.p.m. units. Error bars represent standard deviations of three separate experiments. The protein samples assayed were as follows: *lane 1* - CT histones (2 μg) plus BSA (0.2 μg); *lane 2* - BSA alone; *lane 3* - MonoS pool (0.2 μg) plus BSA and *lane 4* - MonoS pool plus CT histones. The type of core histones present are indicated.

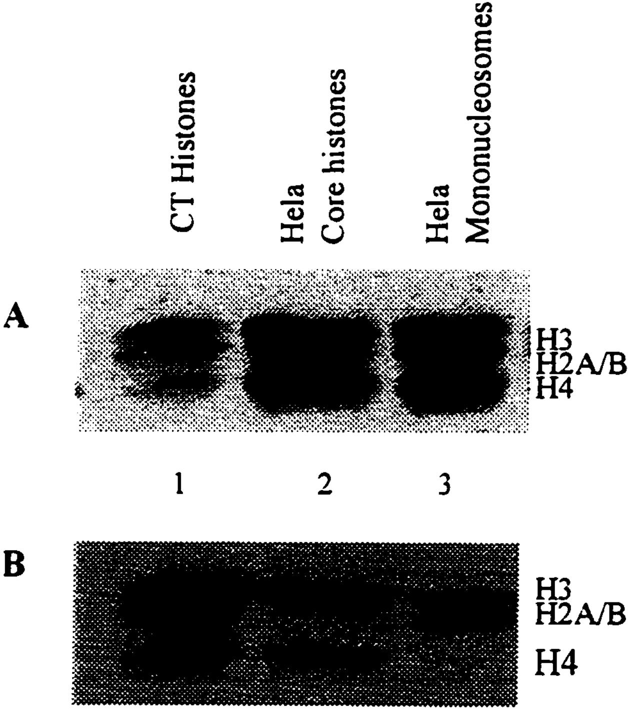

Figure VI-3: HAT activity of the MonoS pool. 0.2 μg of the MonoS pool was incubated with 2 μg each of calf thymus total histones (*lane 1*), core histones from Hela cells (*lane 2*) or mononucleosomes from Hela cells (*lane 3*) and ^{3}H-Acetyl CoA to assay for HAT activity as described in Chapter II (Materials and Methods). The reaction mixtures were analyzed by SDS-PAGE/Coomassie Blue staining and by autoradiograhy of fluorographed SDS-PAGE gels. **Panel A** shows the Coomassie Blue stained gel. and **panel B** shows the corresponding autoradiogram. The contents of each lane and the type of core histones present are indicated.

Taken together these data suggest that the MonoS pool of polypeptides, which includes NF90, possesses HAT activity towards histones that are either free in solution or present within mononucleosomes. In each case H3 was the preferred substrate, however acetylation of H2A/B was seen in CT total histones and Hela mononucleosomes and acetylation of H4 was observed with CT and Hela total histones. It is curious to note that acetlylation of H4 was reduced in the mononcleosome preparation. This is consistent with what is known about nuclear HATs responsible for chromatin remodelling (reviewed in Kuo and Allis, 1998)

In order to determine whether or not NF90 was responsible for the HAT activity present in the MonoS pool, an immunodepletion experiment was performed. Approximately 0.5 µg of the MonoS pool was incubated either with protein G-Sepharose beads alone or with beads that had been precoupled with mouse pre-immune sera or two different mouse polyclonal anti-sera against recombinant NF90 as described in Chapter II (Materials and Methods). Following the period of incubation, the recovered MonoS supernatants were assayed for HAT activity and analyzed by SDS-PAGE/Western blot (Figure VI-4 and 5). HAT activity was quantitated by densitometric analysis of the amount of ^{3}H incorporated into H3 histones (Figure VI-4A and 4B). The MonoS pool incubated with beads precoupled with mouse antibodies NF90-Ab.2 and NF90-Ab.3 retained, respectively, 75% and 55% of its HAT activity when compared to the MonoS sample incubated with beads precoupled with mouse pre-immune sera (compare *lanes 3* and *4* to *lane 2*, Figure VI-4A and 4B). Western blot analysis revealed the absence of NF90 and NF45 in MonoS samples incubated with beads precoupled with mouse antibodies to rNF90 (*NF90Ab.2, NF90Ab.3 - lanes 3, 4*; Figure VI-5), but not with MonoS samples incubated with beads alone or with beads pre-coupled with pre-immune antibodies (*lanes 1,2*; Figure VI-5). Moreover, NF90 and NF45 were present in the immune complexes isolated from the beads pre-coupled with antibodies to rNF90 (*lanes 7, 8*, Figure VI-5). These data suggest that in the absence of any detectable NF90 and NF45, the MonoS pool of polypeptides still possessed more than 55% of the original HAT activity.

In 1995, Brownell and Allis developed a SDS-PAGE gel-based assay to biochemically identify the first histone acetyltransferase in the ciliate protozoa,

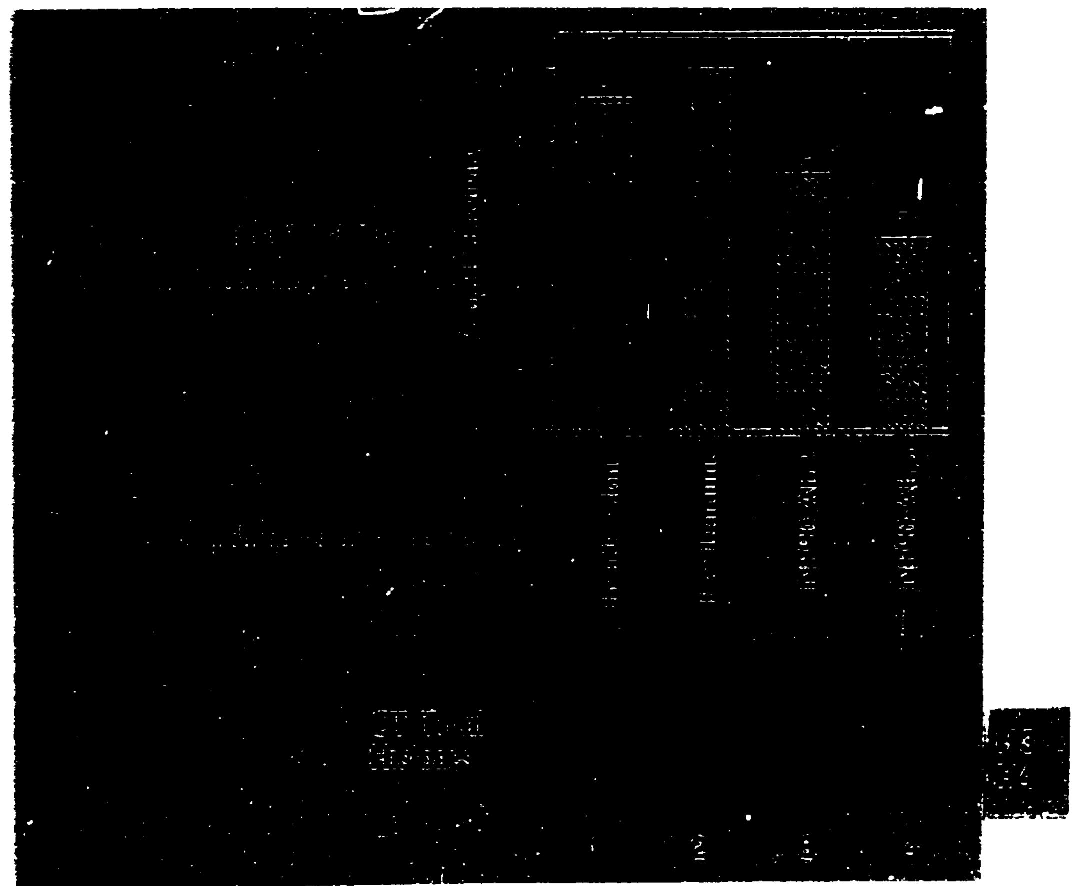

Figure VI-4: HAT assays of MonoS pool immunodepleted for NF90 and NF45. To immunodeplete NF90 from the MonoS pool of proteins, approximately 0.5 μg of MonoS pool were initially incubated with protein-G sepharose beads alone or beads that have been precoupled with antibodies to recombinant NF90 (*NF90Ab.2, NF90Ab.3*) or pre-immune serum. Following incubation, the contents of the recovered supernatant were analyzed by SDS-PAGE/Western blot **(Figure VI-5)** and assayed for HAT activity according to protocol outline in Chapter II (Materials and Methods). HAT activity was quantitated using densitometric analysis of the radioactivity incorporated into histone H3 as it appears on the autoradiogram **(4B)** and expressed as a percentage compared to the HAT activity in the MonoS pool incubated with beads precoupled with pre-immune sera **(4A).**

A) HAT activity remaining of the treated MonoS pool. The MonoS pool incubated with various pre-coupled beads that were assayed for HAT activity are indicated in the graph. Error bars represent standard deviations of from two separate experiments.

B) Gel analysis of the acetylated histones. The lanes contained histones that have been acetylated by the MonoS pool samples that were incubated with the following: *lane 1* - beads alone; *lane 2* - beads precoupled with pre-immune sera; *lane 3* - beads precoupled with mouse antibodies to NF90 (*NF90-Ab.2*) and *lane 4* - beads precoupled with *NF90-Ab.3*. H3 histones used for the densitometric analysis are indicated.

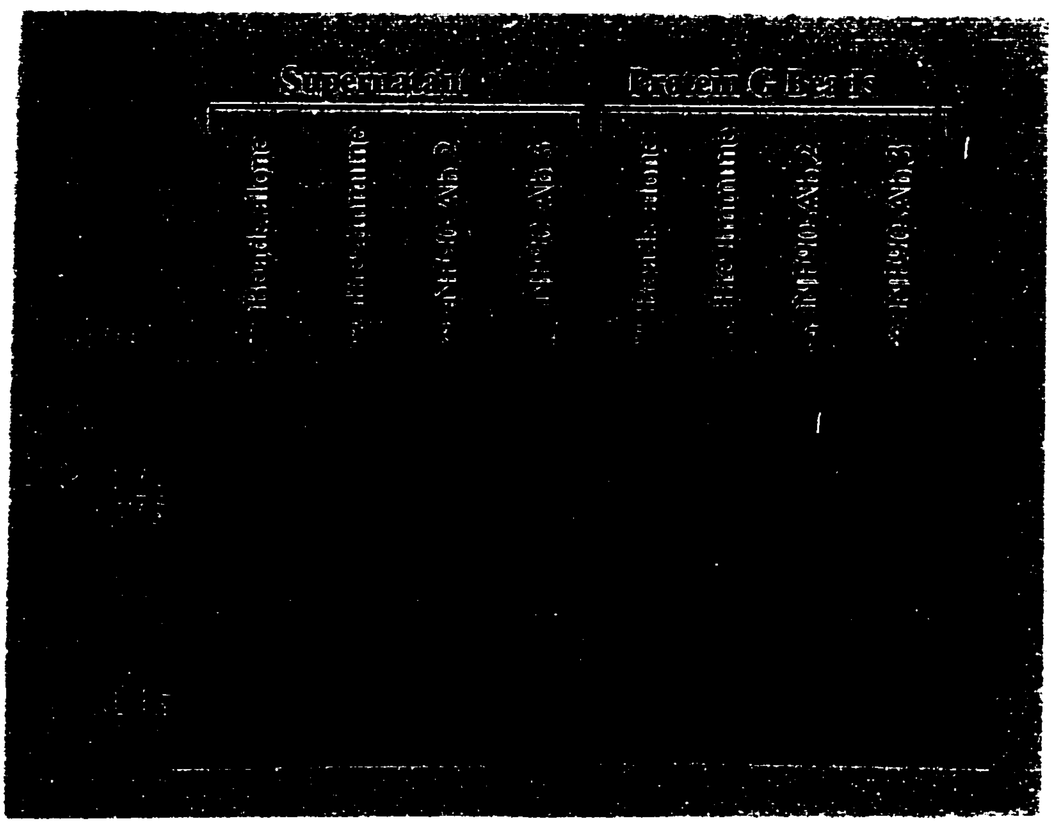

Figure VI-5: Western blot analysis of the MonoS sample and the immune complex of the beads after immunodepletion. An equal amount of the immunodepleted MonoS pool samples used for the HAT assay were fractionated on SDS-PAGE and analyzed by Western blot with rabbit antibodies to recombinant NF90 and NF45. The immune-protein complex isolated from the pre-coupled protein G-Sepharose beads following incubation with the MonoS pool were also analyzed by Western blots with the same antibodies. *Lanes 1-4* contained the MonoS pool supernatant incubated with the antibody precoupled beads as indicated. *Lane 5-8* contained the protein immune-complexes isolated from the pre-coupled protein G-Sepharose beads following incubation with the MonoS pool. The presence of NF90 as p90 and p75 and NF45 are indicated.

Tetrahymena (Brownell and Allis, 1995). This "In-Gel" assay has since been used as the hallmark assay to identify novel histone acetyltransferase proteins. The gel based assay involves fractionation of the protein sample into an SDS-PAGE gel in which total histones had been polymerized into the resolving portion of the gel. Proteins in the gel are denatured by urea and gradually renatured in the presence of detergent, *in situ*. The gel is then incubated with ^{3}H-acetyl CoA and the presence of a HAT enzyme is indicated by ^{3}H-acetate incorporation into histones at a position on the gel corresponding to the migration position of the HAT protein in the gel. Most of the HATs reported in literature to date have been shown to have HAT activity via this method (reviewed in Kuo and Allis, 1998).

We therefore performed the "In-Gel" HAT assays as described in Chapter II (Materials and Methods) with the MonoS pool of proteins, rNF90, rNF45, and a Hela crude cell extract. Incorporation of ^{3}H-acetate was detected in histones corresponding to the migration position of a few polypeptides within the Hela cell extract, but was not present in the MonoS pool or with the rNF90 and rNF45 protein preparations (data not shown). Since polypeptides are fractionated on the gel, it should be noted that this assay would not work if HAT activity required the formation of a multisubunit complex (for example, heterodimerization of NF90 and NF45). Moreover, since the identity of the polypeptides in the Hela crude cell extract is unknown, it is difficult to conclude whether or not they are HAT proteins. Therefore, before we can conclude that NF90 does not have inherent HAT activity, this experiment needs to be repeated using a known HAT (for example, yeast GCN5), in order to ensure that our assay conditions do support HAT activity.

Recombinant NF90, rNF45 or rNF90 plus rNF45 showed no HAT activity in filter binding or gel analysis assays. This is not surprising considering that NF90 and NF45 were expressed in bacterial inclusion bodies and purified via urea treatment. It is possible that the recombinant proteins have not folded properly to yield a catalytically active protein. The addition of purified DNA-PKcs and Ku either with or without DNA to all of the HAT assays containing the MonoS pool or the recombinant proteins did not make a difference in the results (data not shown).

In considering all of our data, we conclude that it is unlikely that NF90 is the

histone acetyltransferase present in the MonoS pool. The concentrations of acetyl-CoA and histones employed for the HAT activity assays were based on published Km values of these substrates for the *Tetrahymena* p55 and yeast GCN5 HATs (Brownell and Allis, 1995; Brownell *et al.*, 1996). Thus, it is unlikely that the HAT activity observed in the MonoS pool was due to an artifact of the *in vitro* assay system. It is therefore possible the HAT activity observed was due to another polypeptide. Purified rat liver eIF-2 or recombinant eIF-2α also showed no HAT activity (data not shown). Again we note that human eIF-2 may have different properties, but to date, we have yet to purify human eIF-2 to homogeneity. Silver stained gels of 0.2 μg of the MonoS pool used in the HAT assays showed predominantly the presence of NF90, p75, NF45 and the eIF-2 proteins (data not shown); therefore, if there are other proteins present that may be a HAT, they are present in very minute amounts.

There are a number of human HATs that have been identified to date. They include pCAF, CBP/p300, $hTAF_{II}250$, human GCN5, and Tip60 (reviewed in Kuo and Allis, 1998). Human GCN5 has recently been shown to interact with Ku70 by yeast two hybrid assays and affinity chromatography columns (Barlev *et al.*, 1998). The same authors showed that phosphorylation of recombinant yeast GCN5 by DNA-PK, resulted in a decrease of HAT activity *in vitro*. A partially purified Hela cell extract contained HAT activity that can be depleted by antibodies to hGCN5. The amount of HAT activity remaining after depletion decreased if the Hela cell fraction was incubated with DNA and ATP. Western blot analysis indicated the presence of DNA-PK in the partially purified Hela cell extract. Taken together, these data suggested that DNA-dependent phosphorylation represses hGCN5 HAT activity (Barlev *et al.*, 1998). Rabbit polyclonal antibodies to GCN5 (kind gift from Dr. C.D. Allis) did not cross react with any polypeptides present in the MonoS pool, suggesting that the HAT activity within this pool is not due to hGCN5 (data not shown).

VI.3: Summary

We conclude that it is unlikely that the HAT activity observed in the MonoS pool can be attributed to NF90. However, a HAT activity is present in our MonoS pool that is capable of acetylating free histones, and histones within mononucleosomes.

CHAPTER VII

RESULTS AND DISCUSSION - PART 5

ELECTROPHORETIC MOBILITY SHIFT ASSAY STUDIES OF DNA-PK COMPLEXES IN CRUDE CELL EXTRACTS

Chapter VII - Electrophoretic mobility shift assay studies of DNA-PK complexes in crude cell extracts[5]

VII.1: Introduction

Electrophoretic mobility shift assay (EMSA) studies with crude cell extracts were initially used to characterize Ku end-binding activity in extracts from *xrs* rodent cells (Getts and Stamato, 1994; Rathmell and Chu, 1994a,b). The observation that certain *xrs* cell lines lacked DNA end-binding activity represented the first step towards identifying DNA-PK as an important protein in DNA double strand break repair and V(D)J recombination (reviewed by Jeggo *et al.*, 1995). To our knowledge, DNA-PK complexes have not been shown in EMSA using crude cell extracts. Since we had observed that chemical cross-linkers were able to stabilize the interaction between highly purified DNA-PKcs and Ku on DNA, we also investigated the possibility of using this method to detect DNA-PK complexes in crude cell extracts.

VII.2: Results and Discussion.

Whole cell extracts were prepared from Hela cells and incubated with the 40 bp DNA probe, either with or without chemical cross-linkers, and the samples were analyzed by electrophoresis on non-denaturing gels as described in Chapter II (Materials and Methods). Hela cell extracts incubated with the 40 bp DNA probe in the **absence** of cross-linkers or unlabeled DNA competitors showed the formation of two protein-DNA complexes (labeled as α and β, *lane 1,* Figure VII-1). The formation of the α and β protein-DNA complexes was reduced by addition of unlabeled linear dsDNA and ssDNA but not closed circular plasmid DNA (data not shown), consistent with the behavior of Ku containing protein-DNA complexes (Paillard and Strauss, 1991; Blier *et al.*, 1993; Rathmell and Chu, 1994a). Moreover, Western blot analysis indicates the presence of Ku in these complexes (data not shown). In the **presence** of BS^3 and absence of any cold competitor DNA, two additional slower migrating complexes were formed with the 40 bp DNA probe and Hela crude cell extracts (labeled δ and γ, *lane 2,* Figure

[5] Figures VII-1 to VII-5 of this chapter are part of a manuscript that has been accepted for publication: Ting, N.S.Y., Chan, D.W., Lintott, L.G., Allalunis-Turner, J., and Lees-Miller, S.P. (1999) *Protein-DNA complexes containing DNA-dependent protein kinase in crude extracts from human and rodent cells.* Radiation Res. **In Press**

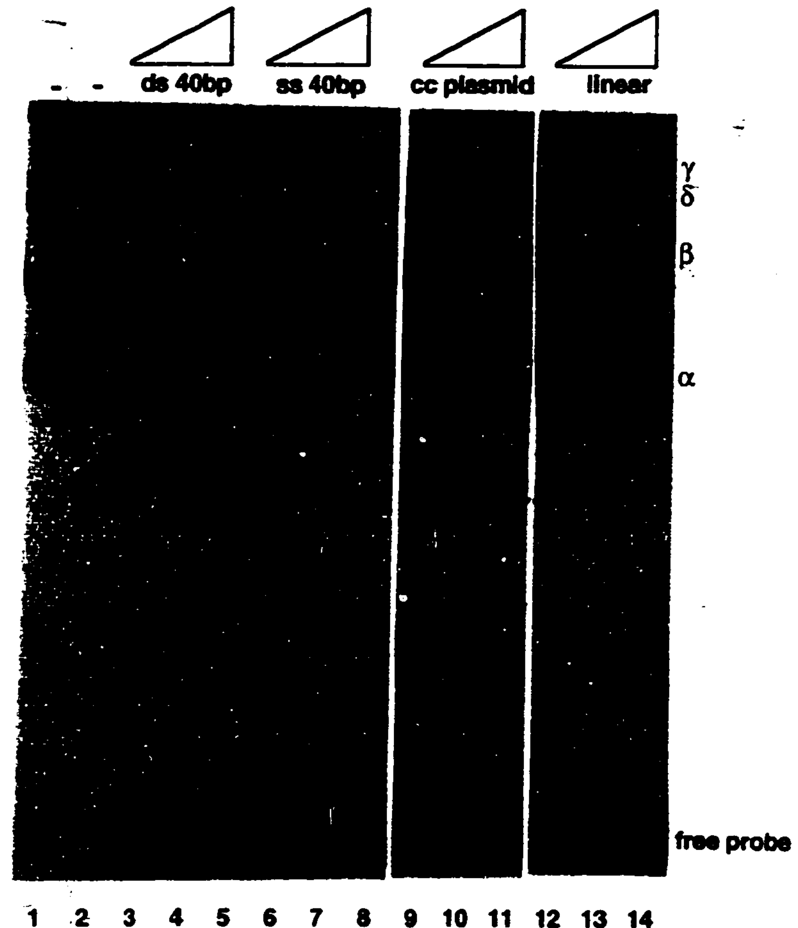

Figure VII-1: Formation of DNA-protein complexes in human cell extracts in the presence of cross-linkers.[6] Whole cell extracts were prepared from Hela cells and incubated with the 40 bp DNA probe with BS^3 (except for *lane 1*) and the formation of protein-DNA complexes was analyzed by EMSA as outlined in Chapter II (Materials and Methods). Unlabeled competitor DNA was added at weight excess to the DNA probe as described in Chapter II (Materials and Methods). All lanes contained 2 μg of Hela cell extract plus BS^3 except in *lane 1* and the following competitor DNA: *lanes 3* to *5* - 5, 20 or 100 fold excess of 40 bp dsDNA; *lanes 6* to *8* - 5, 20, 100 fold excess of 40 bases ssDNA; *lanes 9* to *11* - 20, 100 or 400 fold excess of closed circular plasmid DNA; *lanes 12* to *14* - 20, 100 or 400 fold excess of *Bam*H1 linearized plasmid DNA. The observed protein-DNA complexes are indicated as α, β, δ and γ. All lanes are from the same autoradiogram of a single EMSA experiment.

[6] The experiment for this figure was performed by Lauri G. Lintott.

VII-1). The formation of the slower migrating complexes was reduced with the addition of excess unlabeled ds 40 bp oligonucleotide (*lanes 3-5*, Figure VII-1) and linearized plasmid DNA (*lanes 12-14*, Figure VII-1). Addition of unlabeled ssDNA with 40 bases, slightly reduced the formation of the γ, δ and β complexes (*lanes 6-8*; Figure VII-1), while closed circular plasmid had no effect (*lanes 9- 11*; Figure VII-1). These results are therefore consistent with the DNA binding properties of Ku as previously reported in EMSA studies (Paillard and Strauss, 1991; Blier *et al.*, 1993; Rathmell and Chu, 1994a).

In the presence of BS^3, the protein-DNA complexes α, β and δ formed from whole Hela cell extracts and the 40 bp DNA probe migrated at a position similar to the that of the protein-DNA complexes *A*, *B* and *C* formed with purified DNA-PKcs and Ku with the 40 bp DNA probe under the same conditions (compare *lanes 2* and *6* to lane *8*, Figure VII-2A). This suggested to us that δ and γ complexes may represent DNA-PK-DNA complexes in crude cell extracts. In order to test this possibility, we prepared cell extracts from two human glioma cell lines, MO59J and MO59K for study by EMSA. Even though both cell lines were derived from the same tumor, MO59J contains at least two-hundred fold less DNA-PKcs protein than MO59K (Lees-Miller *et al.*, 1995; Kulesza and Lieber, 1998). In the presence of BS^3, the δ and γ protein-DNA complexes were observed in extracts from MO59K cells, but not in extracts from MO59J cells (compare *lane 6* to *4*, Figure VII-2A). In the absence of BS^3, only the Ku-DNA complexes were formed with the cell extracts from Hela, MO59J and MO59K (*lanes 1,3,5*; Figure VII-2A). In the experiment shown, a faint protein-DNA complex migrating in a similar position as complex γ was present in the MO59K cell extract that had not been treated with BS^3; however, this was not a consistent finding and in general the formation of enhanced and discrete δ and γ protein-DNA complexes were observed only in the presence of BS^3.

Since DNA-PKcs is present in greater abundance in MO59K cells than in MO59J cells, the absence of δ and γ protein-DNA complexes in the MO59J cells suggested that DNA-PKcs was required for their formation. Purified DNA-PKcs, therefore, was added to the extracts from MO59J cells in the EMSA reactions and the formation of δ and γ complexes appeared only in the presence of BS^3 (compare *lane 4* to *lane 2*, Figure VII-2B). It is interesting to note that the δ and γ complexes were formed at the expense of the

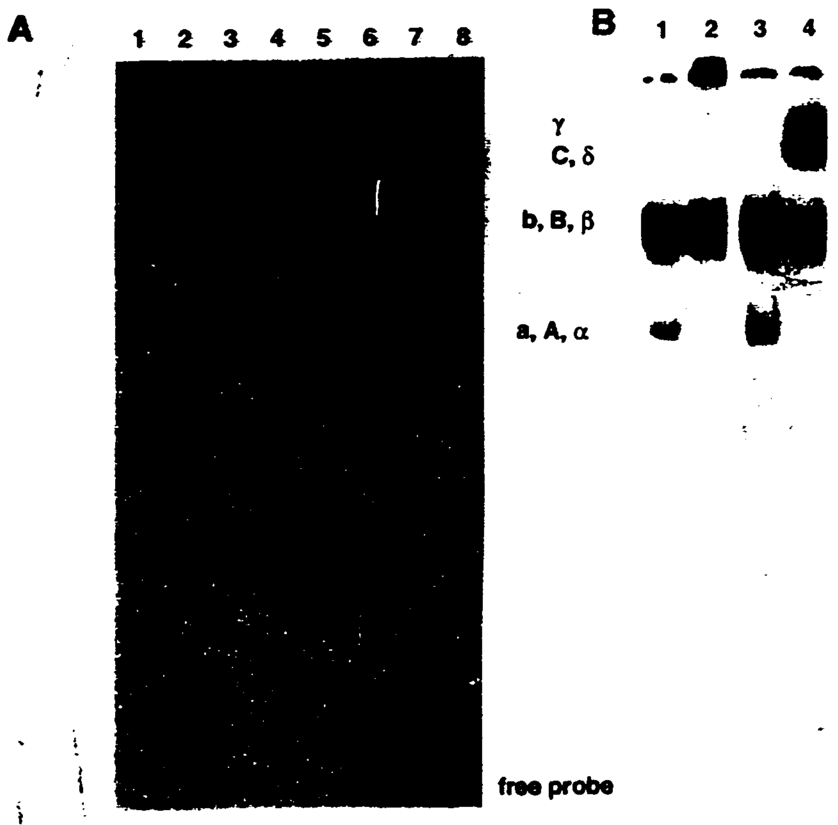

Figure VII-2: A) Formation of δ and γ complexes is absent in MO59J cells.[7] 2 μg of whole cell extracts from Hela, MO59J and MO59K cells were incubated with the 40 bp DNA probe in the presence or absence of BS^3 and analyzed by EMSA as described in Chapter II (Materials and Methods). BS^3 is present only in the even numbered lanes and the contents of each lane are as follows: *lanes 1,2* - Hela; *lanes 3,4* - MO59J; *lanes 5,6* - MO59K. The resulting protein DNA complexes were compared to purified DNA-PKcs (15 ng) and Ku (5 ng) incubated the 40 bp DNA probe with or without BS^3 (*lanes 7* and *8* respectively).
B) Purified DNA-PKcs restored δ and γ complexes in MO59J cells. MO59J whole cell extracts were incubated with the 40 bp DNA probe and the following, for EMSA analysis: *lane 1* - MO59J cell extract alone; *lane 2* - MO59J cell extract with BS^3; *lane 3* - MO59J cell extract with 20 ng DNA-PKcs; *lane 4* - MO59J cell extract with 20 ng DNA-PKcs and BS^3. The protein-DNA complexes formed with purified DNA-PKcs and Ku in the absence of BS^3 are indicated by lower case *a* and *b* and in the presence of BS^3 are indicated by upper case *A, B* and *C*. The protein-DNA complexes formed in the crude cell extracts with BS^3 are indicated by the Greek letters α, β, δ and γ.

[7] The experiment for this figure was performed by Lauri G. Lintott.

Ku-DNA complexes α and β upon addition of DNA-PKcs (compare *lane 3* with *lane 4*, Figure VII-2B). As further evidence for the presence of DNA-PKcs in the δ and γ complexes, purified IgG from rabbit anti-DNA-PKcs serum (DPK1) were shown to prevent the formation of δ and γ complexes (*lanes 4* and *6* in Figure VII-3), while IgG purified from rabbit pre-immune serum had no effect (*lanes 3* and *5*, Figure VII-3). Taken together, these data suggest that DNA-PKcs and Ku are responsible for the formation of the δ and γ complexes.

It is interesting to note that the δ and γ protein-DNA complexes were formed at increasing concentrations of cell extracts at the expense of the α and β Ku-DNA complexes (*lanes 1-4*, Figure VII-4). This is reminiscent of the formation of the multiple protein-DNA complexes seen with increasing concentrations of Ku in EMSA studies (de Vries *et al.*, 1989; Blier *et al.*, 1993). Therefore, one possible explanation for the observed δ and γ complexes could be due to the formation of multiple Ku-DNA-PKcs complexes on DNA. In order to address this possibility, increasing amounts of purified DNA-PKcs were titrated into EMSA reactions containing a concentration of Hela cell crude extracts that showed predominantly α and β complexes. We reasoned that if δ and γ contained only multiple Ku-DNA-PKcs complexes, formation of the δ and γ complexes could be induced from the Ku containing α and β complexes with purified DNA-PKcs. Addition of up to 80 ng of purified DNA-PKcs, however, resulted only in the formation δ complex with concomitant disappearance of the α and β complexes (Figure VII-4, *lanes 6-8*). These data suggested that DNA-PKcs and Ku were not sufficient to form the γ protein-DNA complex and that other proteins are involved.

We had previously shown that the MonoS pool of polypeptides, which contained NF90, or recombinant NF90 (rNF90) alone was able to stabilize the interaction between purified DNA-PKcs and Ku on DNA in the **absence** of any cross-linkers. We therefore asked whether or not protein-DNA complexes formed between purified proteins resembled those protein-DNA complexes formed in crude cell extracts under the same conditions (i.e. in the presence of chemical cross-linkers). Purified DNA-PKcs and Ku incubated with the 40 bp DNA probe formed complex *C* which migrated at a similar position to complex δ formed in crude cell extracts (compare *lane 2* to *lane 4*, Figure VII-

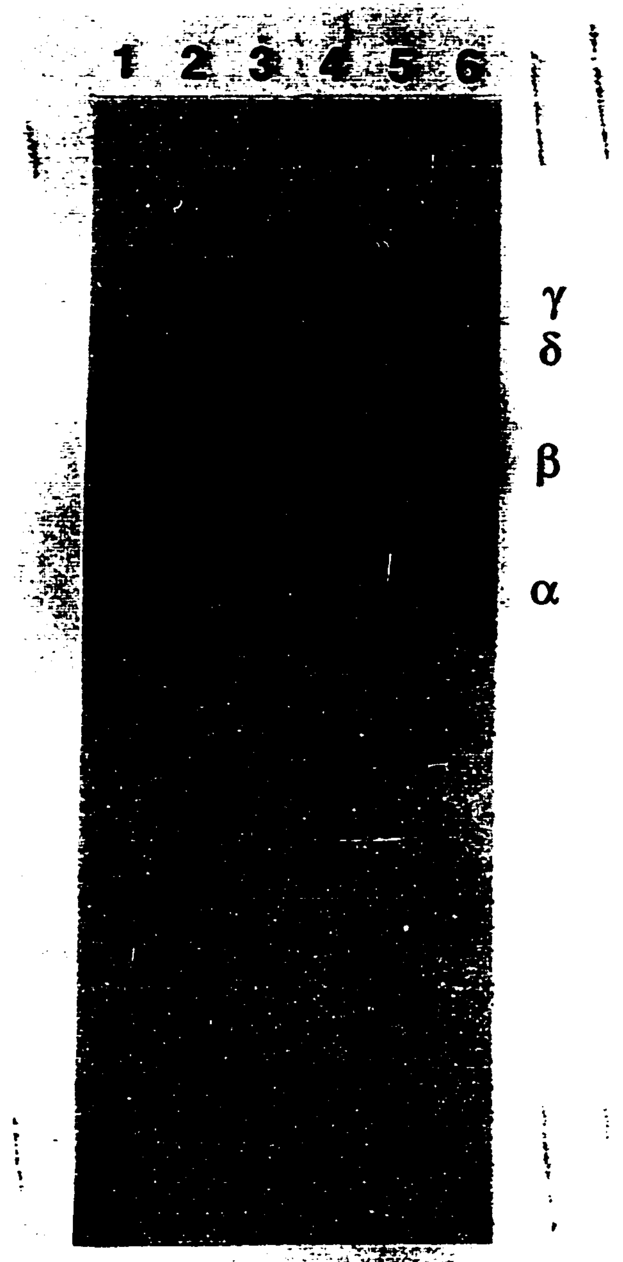

Figure VII-3: Antibodies to DNA-PKcs disrupt formation of δ and γ complex. Hela whole cell extracts were present at 1.5 µg in *lane 1* and at 3 µg in *lanes 2-6*. The whole cell extracts were incubated with the 40 bp DNA probe and BS^3 with the following: *lane 3* - 0.2 µg of IgG purified from rabbit pre-immune serum; *lane 4* - 0.2 µg of IgG purified from DPK1 antiserum; *lane 5* - 0.4 µg of pre-immune IgG; *lane 6* - 0.4 µg of DPK1 IgG. All reaction mixtures were analyzed by EMSA as described in Chapter II (Materials and Methods).

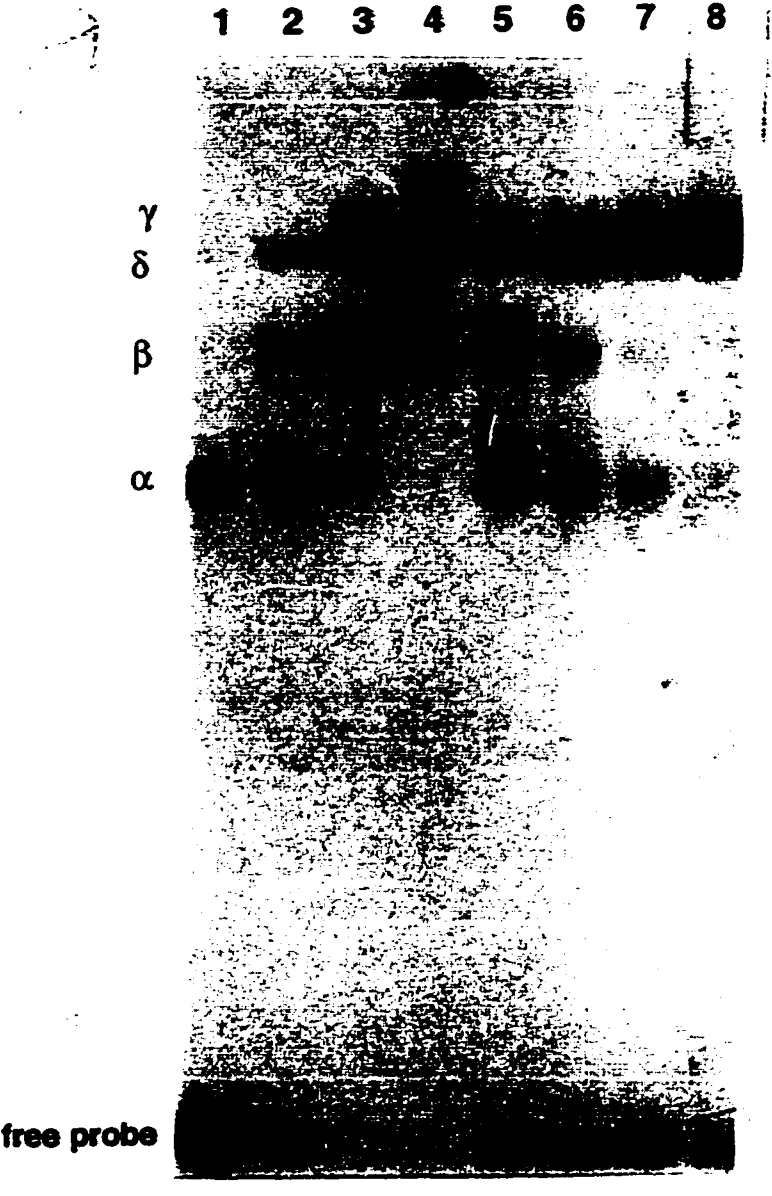

Figure VII-4: The δ and γ protein-DNA complexes may contain DNA-PKcs and other proteins. Different concentrations of whole Hela cell extracts, with or without added purified DNA-PKcs, were treated with BS^3 and assayed in EMSA reactions as described in Chapter II (Materials and Methods). *Lanes 1* to *4* contained 0.5, 1.0, 1.5 and 2.0 μg of Hela whole cell extract, respectively. *Lanes 5* to *8* contained 1.0 μg of whole Hela cell extract with 20, 40, 60 or 80 ng of purified DNA-PKcs, respectively.

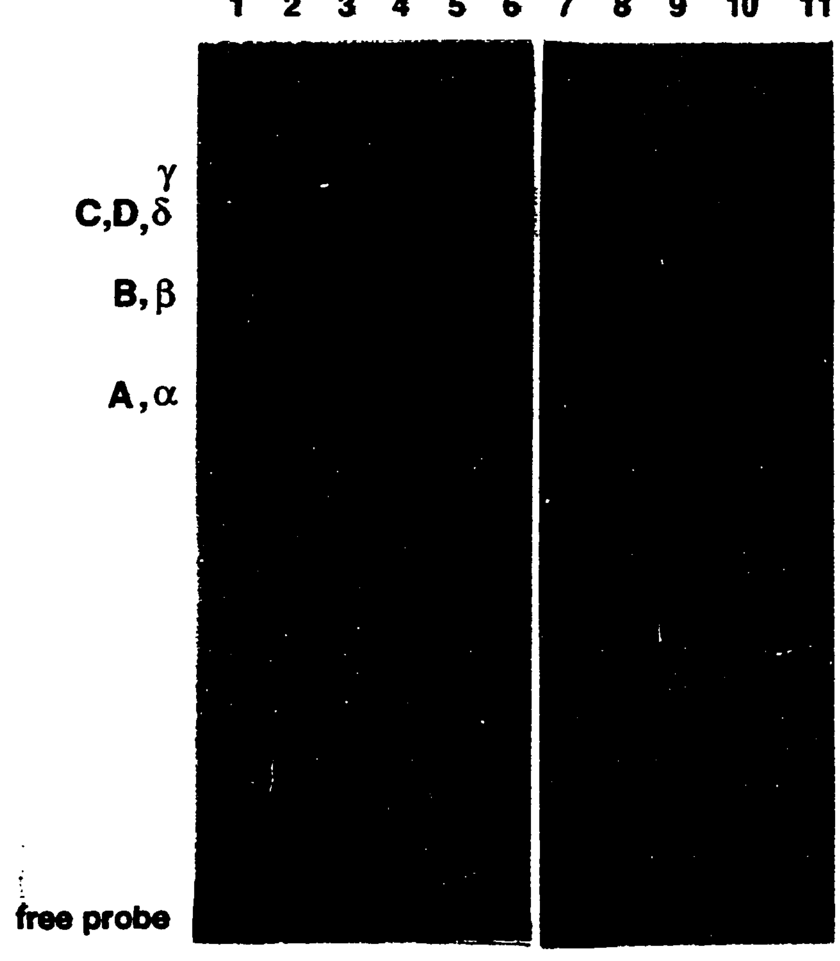

Figure VII-5: Purified DNA-PKcs, Ku, and the MonoS pool of proteins formed a complex that migrates as the δ complex in Hela cells. The contents of each lane are as follows: *lane 1* - free probe; *lane 2* - 2 μg of Hela whole cell extract; *lane 3* - 5 ng Ku; *lane 4*; Ku plus 15 ng DNA-PKcs; *lane 4* - Ku, DNA-PKcs plus 30 ng MonoS pool; *lane 5* - Ku, DNA-PKcs plus 100 ng of recombinant NF90; *lane 7* - 120 ng of MonoS pool. *Lanes 8-11* contained 2 μg of Hela cell extract plus 0, 30, 60 and 120 ng of the MonoS pool. All reaction mixtures were treated with BS^3 and analyzed by EMSA. All lanes shown were from the same exposure of the same gel.

5). Addition of recombinant NF90 or the MonoS pool of polypeptides to purified DNA-PKcs, Ku and the 40 bp DNA probe, enhanced the formation of complex *C* (*lanes 5* and *6*, Figure VII-5), which migrated at a similar position to complex δ in crude cell extracts (compare *lanes 5* and *6* to lane *2*, Figure VII-5). These observations suggested that the γ complex present in crude cell extracts, could not be formed with purified DNA-PKcs, rNF90 or the polypeptides in the MonoS pool under similar EMSA conditions. We then titrated increasing amounts of the MonoS pool of polypeptides into Hela cell extracts containing predominantly α, β, and δ complexes, to determine if these polypeptides could induce the formation of the γ complex. As seen in Figure VII-5, the polypeptides in the MonoS pool did enhance the formation of the δ complex slightly, but did not form the γ complex (*lanes 8-11*, Figure VII-5). Addition of either, the MonoS pool plus purified DNA-PKcs or rNF90 alone, into the same concentration of Hela cell extracts showed similar results (data not shown). This suggested that although rNF90 or the MonoS pool, which contains NF90/45, did not promote the formation of the γ complex in crude cell extracts, these polypeptides may be present in the δ complex. However, if NF90 was present in complex δ, one would expect a change in the migration position of δ complex. One possible explanation why this was not observed is that the predicted molecular mass of DNA-PKcs-Ku-DNA complex is approximately 650 kDa; therefore, due to the resolution limits of our gel system, the presence of a 90 kDa protein may not be sufficient to alter the migration position of such a large complex. In further EMSA experiments, we have been unable to alter the formation of the δ and γ protein-DNA complexes with antibodies to NF90 (data not shown). These antibodies were made to recombinant NF90, and therefore may not recognize native forms of NF90; alternatively, NF90 associated with DNA-PKcs-Ku-DNA complex, may not be accessible to these antibodies. Nonetheless, our data suggest that DNA-PKcs and Ku, along with additional proteins that are not present in the MonoS pool, are required for the formation of the γ complex observed in whole protein extracts from Hela cells.

VII.3: Further discussion and summary

We have demonstrated that several protein-DNA complexes, designated as α, β, δ and γ, can be detected in whole extracts from human cells, when samples are treated with

the chemical cross-linker BS^3 after DNA binding and before analysis, by EMSA (Figure VII-1). The protein-DNA complexes designated as α and β contain Ku; complexes δ and γ require Ku and DNA-PKcs for formation since they are absent from extracts from the DNA-PKcs-deficient radiosensitive human cell line MO59J, and can be abolished by antibodies to DNA-PKcs (Figures VII-2 and VII-3). These results are consistent with our previous EMSA studies with purified proteins, in that a stabilized DNA-PKcs-Ku-DNA complex can be formed only in the presence of chemical cross-linkers. These data suggest that under our EMSA conditions, the interaction between DNA-bound Ku and DNA-PKcs is weak or transient and must be stabilized by addition of cross-linking agents. However, in certain experiments when cell extracts were not treated with cross-linkers, we observed radiolabeled probe retained at the base of the wells, suggesting that the protein-DNA complexes formed may be too large to enter the gel under our electrophoresis conditions. Thus, treatment with cross-linkers is necessary to make the protein-DNA complex more compact to allow resolution under our non-denaturing gel system.

We have previously shown that the polypeptides in the MonoS pool, which contained NF90, or recombinant NF90 alone were able to stabilize the interaction between purified DNA-PKcs and Ku on DNA without cross-linkers. Since NF90 is present in these crude cell preparations (data not shown), why is it that we do not detect an "endogenously-stabilized" DNA-PK-DNA complex under the same EMSA conditions? A possible explanation for not detecting such a complex is that NF90/45 or DNA-PKcs may be complexed with other proteins in a crude cell extract preparation; therefore, DNA-PK or NF90/45 may not be available to specifically interact with each other. Interestingly, addition of the MonoS pool of polypeptides or rNF90 into a concentration of Hela cell extracts that showed predominantly α, β and δ complexes showed slight enhancement of the δ complex but did not induce the formation of γ complex (Figure VII-5). This observation suggests that NF90/45 may further stabilize the interaction of DNA-PKcs and Ku in crude cell extract in the form of the enhanced δ complex. Alternatively, these data also suggest that the formation of the δ and γ complexes requires DNA-PKcs, Ku and possibly additional proteins other than NF90 or those present in the MonoS pool. This EMSA strategy can therefore be used to assay for

other proteins that interact with DNA-PK-DNA complexes. Since DNA-PKcs and Ku are highly abundant in human cells (discussed below), it is likely that the protein(s) responsible for the formation of the δ complex are equally as abundant.

Most other EMSA studies which are performed with crude cell extracts test for the presence of sequence specific DNA binding proteins, such as transcription factors. In our EMSA studies, we are testing for the presence of binding activity for blunt ended 40 bp DNA. DNA-PKcs and Ku have been predicted to be present at approximately 100,000 and about 500,000 molecules per nucleus in human cells, respectively (reviewed in Jackson, 1997; Dynan and Yoo, 1998). Further, Ku has a high affinity for ends of DNA with a K_d of 10^{-10} M (Blier *et al.*, 1993). Hence, based on the abundance of DNA-PKcs and Ku, and the high avidity of Ku for DNA, it is not surprising that DNA-PKcs and Ku containing protein-DNA complexes are easily detected among a milieu of other DNA binding proteins in crude cell extract preparations. This assay can therefore be useful in a number of applications utilizing whole protein extracts from human cells. First, human cells, in particular those with DNA-repair deficiencies, can be rapidly screened for the presence of DNA-PKcs and Ku. Since only one or two µg amounts of cell extracts are needed, this strategy may be more efficient and sensitive than standard Western immunoblot methodologies. Secondly, this method could be used to study DNA-PK-DNA binding behavior following exposure of the cells to DNA damaging agents; more specifically, its DNA binding activity towards different DNA substrates, or how its DNA binding activity is affected by the absence or presence of certain proteins.

Although DNA-PK is known to be involved in NHEJ during DNA dsb repair and V(D)J recombination, very little is known about its exact biochemical function. In this regard, our EMSA strategy of studying DNA-PK complex formation on DNA could be a useful tool to give us further insights on the biochemical role of DNA-PK in these processes.

CHAPTER VIII

FURTHER DISCUSSION AND CONCLUSIONS

Chapter VIII - Further Discussion and Conclusions

VIII.1: Summary

During the initial purification of DNA-PK from Hela cells, several polypeptides that were phosphorylated in a DNA dependent manner were detected in a partially purified fraction that contained DNA-PKcs and Ku. Similar polypeptides were present in a partially purified active kinase fraction, which contains DNA-PKcs, during the purification of DNA-PK from human placenta. We have purified these polypeptides and identified them as the eukaryotic translation initiation factor, eIF-2, and a heterodimeric transcription factor, NF90/45. We have shown that NF90/45 and eIF-2β are novel substrates of DNA-PK *in vitro*.

Since Ku, which we now know is an important regulatory component of DNA-PK, was originally identified as a substrate that was present in a partially purified active DNA-PK fraction, it was important to determine whether or not these substrates also affected DNA-PK activity. We have shown that a pool of proteins which contains NF90/45 and eIF-2 or recombinant NF90 do not affect DNA-PK kinase activity *in vitro*. Since the interaction between DNA-PKcs and Ku on DNA may be important for the function of DNA-PK *in vivo*, we have developed an EMSA strategy to study this interaction *in vitro*. Under these EMSA conditions, highly purified DNA-PKcs and Ku were shown to form a heterotrimeric complex on DNA only in the presence of chemical cross-linker. This data suggested that the interaction between purified DNA-PKcs and Ku on DNA is normally transient or unstable and that this assay could be used to identify factors that could stabilize the interaction between DNA-PK and DNA. Using this strategy, we showed that a pool of polypeptides containing NF90/45 and eIF-2 (called the MonoS pool) was able to stabilize the interaction between DNA-PKcs and Ku on a 40 bp fragment of DNA without the use of chemical cross-linkers. Recombinant NF90 alone was able to stabilize this interaction; moreover, we have localized a region in recombinant NF90 (amino acids 419-571) that was able to support the formation of the stabilized DNA-PK-DNA complex. Antibodies to recombinant NF90 and NF45 immunoprecipitated DNA-PKcs, NF90 and NF45 from crude cell extracts and partially purified placenta protein extract. However, Ku was not detected in these immune

complexes. These data strongly suggest that NF90, which exists as a dimer with NF45, interacts with DNA-PKcs to stabilize the interaction between DNA-PKcs and Ku on DNA.

There is a region of amino acids within NF90 (amino acids 255-278) that bears some amino acid sequence similarity to a highly conserved domain found in all histone acetyltransferase (HAT) proteins identified to date. We therefore tested whether or not NF90 is a histone acetyltransferase by assaying for HAT activity in the MonoS pool of polypeptides. We showed that the MonoS pool of polypeptides possesses histone acetyltransferase activity both towards histones in solution and histones within mononucleosomes. However, we conclude from our immunodepletion and preliminary "In-Gel" HAT assays that NF90 is probably not a histone acetyltransferase. Identification of the HAT protein within this pool of polypeptides would require further chromatographic analysis of the MonoS pool sample.

Finally, we have used the EMSA strategy with chemical cross-linkers to investigate whether or not we could detect the presence of DNA-PK complexes in whole cell extracts. Several protein-DNA complexes containing DNA-PKcs and Ku were formed in the presence of chemical cross-linkers. Although these protein-DNA complexes resembled those protein complexes formed with purified DNA-PKcs, Ku and the MonoS pool or rNF90, we were unable to unequivocally show that NF90 or NF45 was present in these protein-DNA complexes formed in crude cell extracts. We attribute this to a number of possibilities, including the unavailability of suitable antibodies and technical limits of our EMSA system. However, we are certain that one of these protein-DNA complexes, labeled γ in our studies, required DNA-PKcs and Ku, in addition to proteins other than eIF-2 or NF90/45, for its formation. Therefore, this EMSA method could be used to screen for proteins that interact with DNA-PK and study DNA-PK complexes from crude extracts of human cells.

In sum, these results have given insights and suggest further experiments in regards to understanding the function of DNA-PK *in vivo*.

VIII.2: Further Discussions and Future Directions

A) eIF-2 and NF90/45 are *in vitro* substrates of DNA-PK

1 - *Are eIF-2β and NF90/45 in vivo substrates of DNA-PK?*

A number of *in vitro* substrates have been identified for DNA-PK (listed in Table I-1, Chapter I). However, to date, *in vivo* substrates of DNA-PK have not been unequivocally defined. Identification of the *in vivo* targets for DNA-PK represent a crucial step towards understanding of the function of DNA-PK in the cell.

We have identified three *in vitro* substrates for DNA-PK, i.e. NF90, NF45 and eIF-2β. NF90 and NF45 have been found in all human tissues and cells examined to date (Corthesy and Kao, 1994; Kao *et al.*, 1994; Ting and Lees-Miller, unpublished results). Preliminary studies in which NF90 and NF45 were immunoprecipitated from *in vitro* kinase reactions, containing crude Jurkat T-cell extracts, ^{32}P-γATP either, in the presence or absence of CT DNA, indicated that NF90 and NF45 are phosphorylated in a DNA dependent manner (Ting and Lees-Miller, unpublished results). This suggests that NF90 and NF45 may be *in vivo* substrates of DNA-PK. To further test this possibility, NF90 and NF45 could be immunoprecipitated from MO59J and MO59K cells that had been metabolically labeled with ^{32}P-inorganic phosphate. Since MO59J contains at least 200 times less DNA-PKcs protein than MO59K cells (Kulesza and Lieber, 1998), a reduction in the amount of radioactive phosphate incorporated into NF90 or NF45 immunoprecipitated from MO59J compared to MO59K, would suggest that NF90 and NF45 are *in vivo* substrates of DNA-PK. Similar experiments with eIF-2β could be performed; however, due the unavailability of good antibodies to eIF-2, it would be difficult to pursue these types of studies with eIF-2 at this time. Therefore, an initial step towards such studies would be to make antibodies to eIF-2β. To our knowledge, recombinant eIF-2β has not been successfully expressed; thus, the use of purified proteins as antigens for antibody production is likely the best avenue. Alternatively, short (between 10-15 amino acids) synthetic peptide corresponding to amino acid sequence of eIF-2β could also be used as antigens.

2) *Mapping DNA-PK phosphorylation sites on NF90/45 and eIF-2β*

One of the first steps towards investigating the effects of phosphorylation on the

function of a protein is to identify the kinase phosphorylation site(s) on the protein. The phosphorylation site(s) is then mutated, and the mutant form of the protein is expressed to assess the effects on its function, either *in vitro* or *in vivo*. Phosphoamino acid analysis indicates that both serine and threonine residues on NF90, NF45 and eIF-2β are phosphorylated by DNA-PK *in vitro* (Ting and Less-Miller, unpublished results). The amino acid sequence of NF90 and NF45 shows the presence of three S/T-Q and one SQ motifs, respectively; while the amino acid sequence of eIF-2β shows the presence of two TQ sites (Figures X-1 and X-2, Chapter X-Appendix). Preliminary studies in which tryptic phosphopeptides, generated from purified NF90/45 were compared with those generated from rNF90 and rNF45 that had been phosphorylated by DNA-PK *in vitro*, showed very different phosphorylated products on SDS tricine gels (Ting and Less-Miller, unpublished results). This is not surprising considering that NF90 and NF45 were purified from urea denatured bacterial inclusion bodies; thus, the recombinant proteins may not have folded properly and may have different properties compared to the purified proteins. Furthermore, NF90/45 purified from human placenta exists as a heterodimer; therefore, the DNA-PK phosphorylation sites within the individual recombinant NF90 or NF45 subunits may be different from those in the heterodimer. To date, we have not observed any separation between NF90 and NF45 during our purification procedures.

To our knowledge, recombinant eIF-2β has not been successfully expressed in any expression system. Therefore, due to the potential problems of recombinant proteins, future efforts to map DNA-PK phosphorylation sites on eIF-2β and NF90/45 must rely on the use of purified proteins. Alternatively, a different expression system, such as baculovirus and insect cells, could be used to obtain recombinant NF90 or NF45. It would also be a worthwhile pursuit to attempt to co-express NF90 and NF45, simultaneously. This may alleviate some the problems related to the stability of the recombinant proteins encountered throughout this work.

Once the phosphorylation sites on NF90/45 or eIF-2 have been identified, it is important to determine if these are the same sites phosphorylated *in vivo*, before further experiments are undertaken. An initial step towards this goal would be to generate tryptic phosphopeptides from NF90/45 or eIF-2β immunoprecipitated from metabolically ^{32}P-labeled MO59J and MO59K cells, and from *in vitro* phosphorylated NF90/45, and

compare these phosphopeptides by two dimensional high voltage electrophoresis. Similar migration patterns of the phosphopeptides of NF90/45 or eIF-2β isolated from MO59K cells and *in-vitro* phosphorylated NF90/45 or eIF-2 would indicate the same phosphorylation sites. Further, if these phosphopeptides were absent in the NF90/45 or eIF-2β phosphopeptides isolated from MO59J cells, it would strongly suggest that NF90/45 or eIF-2β are *in vivo* substrates of DNA-PK. The results of these experiments would determine whether or not it is worthwhile to pursue further experiments that involve altering the DNA-PK phosphorylation sites on NF90/45 or eIF-2, to eventually investigate the importance of this phosphorylation event in the cell.

B) Interaction of NF90/45 with DNA-PK

1 - *Implications in NHEJ*

Studies with various radiosensitive cell lines and in the yeast system have shown that DNA-PK is required for NHEJ during DNA dsb repair and V(D)J recombination; however, as discussed earlier, the exact biochemical manner in which DNA-PK participates in NHEJ remains uncertain. The DNA substrate used in our EMSA studies is a 40 bp duplex DNA with blunt ends containing no known specific DNA binding sites. The observation that rNF90 alone or the polypeptides in the MonoS pool which contains NF90 was able to stabilize a complex between DNA-PKcs and Ku on DNA suggests this complex may be important at a site of DNA damage *in vivo*. Our results, therefore, may give insights and build a basis for further experiments towards understanding the mechanisms of how DNA-PK may act during repair of DNA double-strand breaks.

Based on what is known about its biochemical properties *in vitro* and on some *in vivo* studies, DNA-PKcs and Ku likely play a structural role in bridging two exposed DNA ends and keeping them synapsed together. These DNA ends may arise during the opening of the hairpin coding ends in V(D)J recombination or during exposure of the genome to ionizing radiation. Maintaining the ends of DNA in close proximity would provide a DNA substrate for the rest of the NHEJ apparatus to enter and biochemically resect the ends to create regions of complementary sequences, leading to the ligation step of the two DNA ends by DNA Ligases. Consistent with this notion, Ku has been shown to stimulate the activity of DNA Ligases I, III, and IV *in vitro*. To date, an interaction

between Ku and the DNA Ligase proteins has not been shown biochemically, but genetic studies in yeast have shown that yeast homologue of DNA Ligase IV (*DNL4*) acts in the same pathway as *YKU80* or *YKU70* (Grawunder *et al.*, 1997; Wilson *et al.*, 1997).

Cells deficient for XRCC4 are radiosensitive and are unable to carry out V(D)J recombination. XRCC4 is a novel protein that bears no significant homology to any other known proteins (Li *et al.*, 1995). XRCC4 associates with, and enhances, DNA Ligase IV activity *in vitro* (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997). EMSA studies have also shown that XRCC4 potentiates the ability of Ku to bind DNA (Leber *et al.*, 1998). The same authors showed that XRCC4 promotes DNA-PK assembly on DNA (Leber *et al.*, 1998). However, the protein-DNA complexes claimed to contain XRCC4, DNA-PKcs, Ku and DNA were not very discrete, with "smeary" and weak DNA probe signals. The potential interaction between DNA-PK and XRCC4 was not further substantiated by any other biochemical means (Leber *et al.*, 1998). Therefore, the evidence for an interaction between DNA-PK and XRCC4 is, at present, weak. These data, however, suggest that XRCC4 acts as a "bridging factor" that associates with Ku and DNA Ligase IV to facilitate DNA joining during NHEJ *in vivo*.

Based on our EMSA and immunoprecipitation data, we proposed that the interaction of NF90/45 with DNA-PKcs is necessary to enhance the association of DNA-PKcs with Ku on DNA, *in vitro*. Whether or not NF90/45 remains associated with DNA-PKcs once it is localized with Ku on DNA remains to be determined. Therefore, NF90/45 may have a role, similar to the role proposed for XRCC4, in potentiating and stabilizing a DNA-PK complex at the ends of dsDNA breaks *in vivo*. In this regard, we have obtained recombinant XRCC4 used by Leber *et al.*, (1998) and compared its ability to stabilize an interaction between DNA-PKcs and Ku on DNA with the ability of rNF90 to stabilize the same interaction. Incubation of up to 4 μg of recombinant XRCC4 with purified DNA-PKcs, Ku and the 40 bp DNA probe did not induce the formation of a stabilized protein-DNA complex similar to the protein-DNA complex formed by incubation of 100 ng rNF90 with DNA-PKcs and Ku under similar EMSA conditions (Ting and Lees-Miller, unpublished results). It would be interesting to perform the same experiment using purified human XRCC4 and NF90/45. Nevertheless, these observations suggest that NF90 may also be an important factor in stabilizing the

interaction between DNA-PKcs and Ku at the ends of DNA during NHEJ.

The stabilization of DNA-PKcs and Ku on DNA by NF90/45, may be important during NHEJ for a number of reasons. First, it may further stabilize the biochemical configuration of the intermolecular bridge between two DNA ends formed by Ku and DNA-PKcs. This stabilized protein-DNA complex would make a more favorable substrate for DNA Ligases to eventually join the broken DNA ends. This possibility could be easily tested by adding NF90/45 into *in vitro* plasmid religation assays with DNA Ligases/Ku or *in vitro* recombination assays with the RAG1, RAG2, HMG1, HMG2 and DNA Ligases (Ramsden and Gellert, 1998).

Secondly, DNA-PKcs associated with Ku on DNA may be important for DNA-PKcs to recruit other proteins to the site of DNA end joining. It is also possible that NF90/45 alone, or in association with DNA-PKcs, may be responsible for recruiting other proteins to the sites of DNA damage. One very attractive candidate is a histone acetyltransferase protein. Recruitment of HAT may be important in establishing a proper heterochromatin structure during DNA end-joining. It has been reported that DNA-PK phosphorylates and represses the activity of histone acetyltransferase, hGCN5 (Barlev *et al.*, 1998). Hence, it is likely that association of DNA-PK with a HAT protein is important for its function *in vivo*. To date, NF90/45 has not been shown to interact with any other proteins. One way to identify proteins that interact with NF90 or NF45 would be to carry out a yeast two hybrid screen using a human cDNA library and NF90 or NF45 as the bait.

Lastly, a stabilized DNA-PKcs-Ku complex on DNA in the presence of NF90/45 may stimulate DNA-PK phosphorylation of other proteins. This phosphorylation event may be important in signaling and regulating the activity of the target protein during NHEJ. Although the presence of NF90/45 does not stimulate DNA-PK activity towards the SQE peptide, NF90/45 may stimulate DNA-PK to phosphorylate other proteins when these proteins are all assembled in a multiprotein complex *in vivo*. An excellent candidate would be XRCC4, which is the only protein thus far known to be required for V(D)J recombination and NHEJ, that is shown to be phosphorylated by DNA-PK, *in vitro*. PARP has been shown to stimulate DNA-PK activity towards RPA and p53 *in vitro* (Ruscetti *et al.*, 1998). Thus, this possibility could be easily tested in an *in vitro*

kinase assay using purified DNA-PKcs and Ku with recombinant XRCC4 and NF90/45.

2 - *Implications in the control of transcription.*

NF90 and NF45 were initially purified from a multiprotein complex that associated with the antigen receptor response element (ARRE) in the enhancer region of the interleukin-2 (IL-2) gene (Corthesy and Kao, 1994; Kao *et al.*, 1994). IL-2 is a cytokine important for differentiation and proliferation of T-cells during lymphocyte development and immune responses (Crabtree, 1989). The association of the protein complex with ARRE is observed only in nuclear extracts from Jurkat T-cells that had been stimulated with phorbol myristate acetate (PMA) and ionomycin. In an *in vitro* transcription assay, using nuclear extracts from activated Jurkat T-cells, antibodies to NF90 and NF45 inhibited transcriptional activity of a reporter construct containing the IL-2 promoter. NF90 and NF45 were therefore proposed to be transcription factors involved in the expression of the IL-2 upon activation of T-cells (Kao *et al.*, 1994). However, since their discovery, very little is known about how NF90 and NF45 is involved in the expression of IL-2.

There are a number of reports to suggest a role for DNA-PK in the control of transcription of certain genes. First, DNA-PKcs and Ku have been found to associate with the RNA polymerase II holoenzyme complex that includes basal transcription factors and DNA repair proteins (Maldanado *et al.*, 1996). Secondly, DNA-PK inhibits transcription of certain genes by RNA polymerase I (Kuhn *et al.*, 1995; Labhart, 1995). Further, DNA-PK activity, stimulated by the NRE1 element on the MMTV promoter, is required to repress transcription by RNA-polymerase II from the MMTV promoter (Giffin *et al.*, 1996; 1997). However, the exact biochemical function of DNA-PK in the control of transcription remains to be determined.

One of the significant phenotypes of the knockout mice for both DNA-PKcs (*slip*) and Ku70 is the propensity of these mice to develop T-cell lymphomas (Li *et al.*, 1998; Jhappan *et al.*, 1997). Although the exact molecular mechanism behind the neoplastic conversion has yet to be determined, it is possible that aberrant expression of IL-2 could result in the observed phenotype, since IL-2 is required for T-cell proliferation and differentiation. Overexpression of IL-2 has been implicated as a causal factor or at least

one of the factors involved in maintaining certain T-cell lymphomas such as non-Hodgkin lymphoma and adult T-cell leukemia (Duprez *et al.*, 1985; Nagarkatti *et al.*, 1994; Hassuneh *et al.*, 1997). Therefore, DNA-PKcs and Ku may be involved in regulating IL-2 expression during T-cell development.

Based on our results, there are a number of possibilities as to how DNA-PK may function in the expression of IL-2. It has been previously shown that the addition of phosphatase reduces the ARRE binding activity of the nuclear extracts isolated from activated Jurkat T-cells (Corthesy and Kao, 1994). Therefore, phosphorylation of NF90/45 may be an important signaling mechanism for association with the ARRE sequence. To date, DNA-PK is the first kinase shown to phosphorylate NF90/45 *in vitro*. In order to determine if phosphorylation of NF90 and NF45 by DNA-PK is important for its function *in vivo*, DNA-PK phosphorylation sites on NF90 and NF45 must be identified. These sites could then be altered, for example from serine or threonine to alanine, and constructs expressing NF90 or NF45 containing the altered phosphorylation sites could be transfected into Jurkat T-cells. These cells would be stimulated by PMA and ionomycin, and ARRE-binding activity could be assessed by EMSA. Ideally, this experiment should be performed in NF90 or NF45 deficient cell lines; however, since no such cell lines are available at this time, the NF90/45 mutant constructs would need to be overexpressed to obtain any interpretable results.

If the phosphorylation of NF90/45 by DNA-PK is important during IL-2 expression, how might DNA-PK be stimulated? Our immunoprecipitation and EMSA data have shown that NF90/45 interacts with DNA-PKcs, and this interaction is required to stabilize the interaction between DNA-PKcs and Ku on DNA. It is possible that DNA-PK is activated once it is co-localized with NF90/45 at the ARRE sequence in the absence of DNA ends. It has been shown that co-localization of the glucocorticoid receptor (GR) and DNA-PK on the NRE1 element in the MMTV promoter enhanced phosphorylation of GR by DNA-PK, *in vitro* (Giffin *et al.*, 1997). In order to investigate the possibility that DNA-PK and NF90/45 may assemble at the ARRE site, a closed circular plasmid containing the ARRE sequence could be used in competition assays using EMSA studies with purified DNA-PK, Ku and NF90/45. This would ensure that any competition observed would be due to specific binding to the ARRE sequence and

not due to DNA end-binding activity of Ku. The same closed circular plasmid with the ARRE sequence could also be tested to determine whether DNA-PK activity is stimulated by the ARRE sequence in the presence and absence of NF90/45. DNA-PK activity has been previously shown to be stimulated by the NRE1 sequence when placed within a closed-circular plasmid (Giffin *et al.*, 1996). As further evidence that DNA-PK assembles with NF90/45 at the ARRE site, antibodies to DNA-PKcs, Ku80 or Ku70 could be added to nuclear extracts isolated from activated Jurkat T-cells in EMSA studies for ARRE binding. A reduced signal for ARRE binding activity in the presence of the antibodies to DNA-PK would suggest that DNA-PK is present in the multiprotein complex associated with the ARRE sequence.

It is also possible that association of DNA-PK with NF90/45 at the ARRE site is required to stimulate DNA-PK activity towards other factors that are important in the regulation of transcription. The HSF1 transcription factor has been shown to stimulate DNA-PKcs activity towards the CTD of RNA polymerase II, *in vitro* (Peterson *et al.*, 1995a). Although NF90/45 has no effect on DNA-PK activity towards the SQE peptide, it would be interesting to determine if NF90/45 affects DNA-PK activity towards the CTD of RNA polymerase II or other basal transcriptional machinery factors such as TBP of TFIID. Phosphorylation of CTD of RNA polymerase II and TBP, has been proposed to be one of the possible mechanisms by which DNA-PK may regulate transcription (Labhart, 1996; Chibazakura *et al.*, 1997).

In order to investigate a possible role of DNA-PK in the expression of IL-2 *in vivo*, the following experiments could be pursued. First, various cells of lymphoid origin from DNA-PKcs-, Ku80-, or Ku70-deficient mice could be used to test for IL-2 expression and ARRE binding activity, upon treatment of the cells with PMA and ionomycin. If there is a difference in these activities in any of the Ku deficient cell lines, the cDNA for Ku80 or Ku70 should be transfected into these cells in order to confirm the involvement of Ku in the regulation of IL-2 expression. Secondly, Ku80 or Ku70 could be overexpressed in Jurkat T-cells; any change in the levels of IL-2 transcripts or protein would suggest the involvement of Ku80 or Ku70. Overexpression of Ku70 has been previously shown to decrease the expression of the hsp70 (Yang *et al.*, 1996). To our knowledge, full length cDNA for DNA-PKcs is presently unavailable due in part to the

large size of the DNA-PKcs gene (over 13 kb); hence, it is not possible to pursue similar experiments with DNA-PKcs at this time.

Finally, we have also shown that a region between amino acids 419 and 591 in recombinant NF90 was able to stabilize the interaction between DNA-PKcs and Ku on DNA. To determine if this region is important for interaction *in vivo*, a vector encoding this region could be transfected into and overexpressed in Jurkat T-cells. If this region is important, the overexpressed NF90 deletion fragment would be expected to titrate out DNA-PKcs, and therefore have a dominant-negative effect on the interaction between DNA-PKcs and NF90. IL-2 expression or ARRE binding could used to monitor the effects. On the other hand, the NF90 deletion fragment could further stabilize the interaction between NF90 and DNA-PK resulting in enhanced transcriptional activity. In either case, the results could be compared to a control vector that expresses the N-terminal region of NF90 (amino acids 1-314) which showed no stabilization activity in our EMSA studies. Alternatively, amino acid residues within the region 419-591 that are known to be important for protein-protein interactions, for example, hydrophobic amino acids, could be mutated and tested in EMSA studies in order to determine if these mutated forms of NF90 are still able to stabilize the interaction between DNA-PKcs and Ku on DNA. Constructs containing these mutated forms of NF90 that do not retain the "stabilizing activity" could be expressed in Jurkat T-cells as further, perhaps better, controls.

C) Further considerations

The quintessential experiment to determine the importance of NF90 and NF45 in the eukaryotic cell is to create cell lines that lack NF90 and NF45 by targeted disruption of their respective genes. These experiments could be performed with Jurkat T-cells or Hela cells. Alternatively, other experiments aimed at altering the expression or activity of NF90/45 could also be pursued. For example, expression of antisense RNA could be used to inhibit production of NF90 and/or NF45. Cells inactivated for NF90 and/or NF45 could be tested for sensitivity to ionizing radiation or subjected to transient recombination assays to determine the importance of NF90/45 in NHEJ for DNA dsb repair or V(D)J recombination. Furthermore, these cells could be tested for IL-2 expression and ARRE

binding in EMSA studies. To date, genes encoding NF90 and NF45 have not been identified in mouse or other lower eukaryotes. The identification of these genes would be a first step to determine the importance of NF90 and NF45 in the development of a whole organism.

If NF90 or NF45 is important in DNA dsb repair or V(D)J recombination, it is possible that there are cell lines that have naturally occurring mutations for NF90 and NF45. A number of radiosensitive human cell lines have been identified. One human cell line, called 180BR, is a lymphoblastoid cell line that was established from a patient with leukemia who showed an adverse response to radiotherapy. These cells are extremely radiosensitive, but were recently shown to have normal levels of PARP, Ku, DNA-PKcs and ATM (Lu *et al.*, 1998). Furthermore, bone marrow and fibroblast cells from one human SCID patient have been shown to be radiosensitive; however, these cells have wild type DNA-PK and Ku end-binding activity (Nicolas *et al.*, 1996). Of great interest, it has been recently shown that these cells were also unable to carry out V(D)J recombination, but displayed wild type genetic markers for XRCC4, DNA Ligases I and IV (Nicolas *et al.*, 1998). Interestingly, T-cells from another human SCID condition have been demonstrated to have lowered expression of IL-2 protein; it was further shown that transcription activity of reporter constructs containing ARRE sites transfected into these cells displayed 2 to 5 fold decrease in activity compared to wild type cells (Castigli *et al.*, 1993). It would be interesting to determine if these cells from this SCID condition are sensitive to ionizing radiation. The genetic defect(s) in all of these cells has not been identified to date. Finally, of the eleven genetic complementation groups initially established to study DNA repair, the genetic defect in at least three groups is presently unknown (Zdzienicka, 1996). Therefore, it would be important to check for the presence of NF90 and NF45 in all of these cell lines.

VII.3: Conclusions

At the beginning of this project five years ago, very little was known about the function of DNA-PK in the eukaryotic cell. Today, studies from various radiosensitive cell lines and mice knockout models for Ku80, Ku70 and DNA-PKcs have shown that DNA-PK is required for the process of non-homologous end-joining during DNA double-

strand break repair and V(D)J recombination. There is also evidence to suggest a role for DNA-PK in the cellular response pathway to DNA-damage and the regulation of transcription of certain genes. However, still very little is known about the biochemical function of DNA-PK within these processes. In this regard, the findings presented in this thesis have in part contributed to our understanding and provided a basis for future experiments to better understand the role of DNA-PK within the eukaryotic cell.

CHAPTER IX

BIBLIOGRAPHY

Chapter IX - Bibliography

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CHAPTER X

APPENDIX

Chapter X- Appendix

eIF-2α

1 MPGLSCRFYQ HKFPEVEDVV MVNVRSIAEM GAYVSLLEYN NIEGMILLSE
51 LSRRRIRSIN KLIRIGRNEC VVVIRVDKEK GYIDLSKRRV SPEEAIKCED
101 KFTKSKTVYS ILRHVAEVLE YTKDEQLESL FQRTAWVFDD KYKRPGYGAY
151 DAFKHAVSDP SILDSLDLNE DEREVLINNI NRRLTPQAVK IRADIEVACY
201 GYEGIDAVKE ALRAGLNCST ENMPIKINLI APPRYVMTTT TLERTEGLSV
251 LSQAMAVIKE KIEEKRGVFN VQMEPKVVTD TDETELARQM ERLERENAEV
301 DGDDDAEEME AKAED

eIF-2β

1 MSGDEMIFDP TMSKKKKKKK KPFMLDEEGD **TQ**TEE**TQ**PSE TKEVEPEPTE
51 DKDLEADEED TRKKDASDDL DDLNFFNQKK KKKKTKKIFD IDEAEEGVKD
101 LKIESDVQEP TEPEDDLDIM LGNKKKKKKN VKFPDEDEIL EKDEALEDED
151 NKKDDGISFS NQTGPAWAGS ERDYTYEELL NRVFNIMREK NPDMVAGEKR
201 KFVMKPPQVV RVGTKKTSFV NFTDICKLLH RQPKHLLAFL LAELGTSGSI
251 DGNNQLVIKG RFQQKQIENV LRRYIKEYVT CHTCRSPDTI LQKDIRLYFL
301 QCETCHSRCS VASIKTGFQA VTGKRAQLRA KAN

eIF-2γ

1 MAGGEAGVTL GQPHLSRQDL TTLDVTKLTP LSHEVISRQA TINIGTIGHV
51 AHGKSTVVKA ISGVHTVRFK NELERNITIK LGYANAKIYK LDDPSCPRPE
101 CYRSCGSSTP DEFPTDIPGT KGNFKLVRHV SFVDCPGHDI LMATMLNGAA
151 VMDAALLLIA GNESCPQPQT SEHLAAIEIM KLKHILILQN KIDLVKESQA
201 KEQYEQILAF VQGTVAEGAP IIPISAQLKY NIEVVCEYIV KKIPVPPRDF
251 TSEPRLIVIR SFDVNKPGCE VDDLKGGVAG GSILKGVLKV GQEIEVRPGI
301 VSKDSEGKLM CKPIFSKIVS LFAEHNDLQY AAPGGLIGVG TKIDPTLCRA
351 DRMVGQVLGA VGALPEIFTE LEISYFLLRR LLGVRTEGDK KAAKVQKLSK
401 NEVLMVNIGS LSTGGRVSAV KADLGKIVLT NPVCTEVGEK IALSRRVEKH
451 WRLIGWGQIR RGVTIKPTVD DD

Figure X-1: Amino acid sequences of the α, β and γ-subunits of human eIF-2. For eIF-2β the potential DNA-PK phosphorylation sites are indicated as bold letters and the polylysine blocks are underlined (Ernst *et al.*, 1987; Pathak, *et al.*, 1988; Gaspar *et al.*, 1994).

NF45

1 MRGDRGRGRG GRFGSRGGPG GGFRPFVPHI PFDFYLCEMA FPRVKPAPDE

51 TSFSEALLKR NQDLAPNSAE QASILSLVTK INNVIDNLIV APGTFEVQIE

101 EVRQVGSYKK GTMTTGHNVA DLVVILKILP TLEAVAALGN KVVESLRAQD

151 PSEVLTMLTN ETGFEISSSD ATVKILITTV PPNLRKLDPE LHLDIKVLQS

201 ALAAIRHARW FEENA**SQ**STV KVLIRLLKDL RIRFPGFEPL TPWILDLLGH

251 YAVMNNPTRQ PLALNVAYRR CLQILAAGLF LPGSVGITDP CESGNFRVHT

301 VMTLEQQDMV CYTAQTLVRI LSHGGFRKIL GQEGDASYLA SEISTWDGVI

351 VTPSEKAYEK PPEKKEGEEE EENTERTTSR RGRRKHGNSG VTFPSLLFLP

401 KGKTGA

NF90

1 MRPMRIFVND DRHVMAKHSS VYP**TQ**EELEA VQNMVSHTER ALKAVSDWIH

51 EQEKGSSEQA ESDNMDVPPE DDSKEGAGEQ KTEHMTRTCR GVMRAGPGGQ

101 SASYSRGTWI WSWCCCVRRS PQPALLDKVA DNLAIQLAAV TEDKYEILQS

151 VDDAAIVIKN TKEPPLSLTI HLTSPVVREE MEKVLAGETL SVNDPPDVLD

201 RQKCFAALAS LRHAKWFQAR ANGLKSCVIV IRVLRDLCTR VPTWGPLRGW

251 PLEL***LCEKSI*** ***GTANRPMGAG*** ***EALRRVLE***CL ASGIVMPDGS GIYDPCEKEA

301 TDAIGHLDRQ QREDI**TQ**SAQ HALRLAAFGQ LHKVLGMDPL PSKMPKKPKN

351 ENPVDYTVQI PPSTTYAITP MKRPMEEDGE EKSPSKKKKK IQKKEEKAEP

401 PQAMNALMRL NQLKPGLQYK LV**SQ**TGPVHA PIFTMSVEVD GNSFEASGPS

451 KKTAKLHVAV KVLQDMGLPT GAEGRDSSKG EDSAEETEAK PAVVAPAPVV

501 EAVSTPSAAF PSDATAENVK QQGPILTKHG KNPVMELNEK RRGLKYELIS

551 ETGGSHDKRF VMEVEVDGQK FQGAGSNKKV AKAYAALAAL EKLFPDTPLS

601 PLMPTKRREP QYPSEGDRNL LLSHITLASA WEAPCTTKCP HPPTFEGGEE

651 AGRSGDEGAG EDLVAPTMEA T

Figure X-2: Amino acid sequences of human NF45 and NF90. The potential DNA-PK phosphorylation sites on NF45 and NF90 are indicated in bold letters. For NF90, the region with the amino acid similiarity to histone acetyltransferase motif A is indicated by bold and italicised letters (amino acids 255-278). The deletion rNF90 fragment that is able to stabilize the interaction between DNA-PKcs and Ku on DNA in EMSA is underlined (amino acids 417-591) (Kao and Corthesy, 1994; Kao *et al.*, 1994).