ROLE OF NUCLEAR MATRIX PROTEIN C1D IN REGULATION OF TRANSCRIPTION

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AUGUST 2001

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ABSTRACT

ROLE OF THE NUCLEAR MATRIX PROTEIN C1D IN REGULATION OF TRANSCRIPTION

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M.S in Molecular Biology and Genetics

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The nuclear matrix protein C1D is an activator of DNA-dependent protein kinase (DNA-PK), which is involved in non-homologous end joining and V(D)J recombination. Moreover, the nuclear matrix proteins C1D was shown to be phosphorylated *in vitro* by DNA-PK and its mRNA and protein levels have been demonstrated to be induced upon γ-irradiation. The available information suggests that C1D may play a role in DNA DSB-response pathways by targeting DNA-PKcs to nuclear matrix and matrix-associated DNA where DNA-PK may regulate several cellular processes. In an attempt to identify the biological function of C1D, the yeast two-hybrid system was employed and C1D was found to interact with a partial clone of TAFI 48, which is a subunit of the promoter selectivity factor SL1 required for the accurate initiation of transcription from the human ribosomal promoter. The possibility of an interaction between C1D and TAFI 48 was potentially important as DNA-PK was found to be capable of repressing Polymerase I transcription *in vitro.*

In this study, it was shown that C1D and TAFI 48 interact specifically in yeast, *in vitro* and in mammalian cells. It ıs belıeved that this interaction could have potential roles in regulating the repression of transcription from rDNA by DNA-PK in response to DNA double-strand breaks.

Key Words: C1D, DNA-PK, DNA Double-strand break, TAFI 48.

ÖZET

NÜKLEER MATRİKS PROTEİNİ C1D'NİN TRANSKRİPSİYONUN REGÜLE EDİLMESİNDEKİ ROLÜ

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Nükleer matriks proteini C1D, DNA uçlarının doğrudan bağlanmasında ve V(D)J rekombinasyonunda rol oynayan DNA bağımlı protein kinazın (DNA-PK) bir aktivatörüdür. Ayrıca nükleer matriks proteini C1D'nin , DNA-PK tarafından *in vitro* şartlarda fosforlanabildiği ve γ irradyasyonu sonrası mRNA ve protein seviyesinin arttığı gösterilmiştir. Elimizdeki bilgiler çift sarmal DNA kırıklarına tepki olarak C1D'nin DNA-PK'yı nükleer matrikse veya matrikse ilintili DNA'ya, birçok hücresel olayı kontrol etmek amacıyla yönlendirebileceğini önermektedir. C1D'nin biyolojik fonksiyonunu bulmaya yönelik bir denemede maya ikili hibrid sistemi uygulanmış ve C1D'nin insan ribosomal promotorlarından transkripsiyonun doğru başlaması için gerekli olan SL1'nın bir alt ünitesi olan TAFI 48'in bir kısmını kodlayan bir klon ile etkileştiği bulunmuştur. DNA-PK Polimeraz I transkripsiyonunu *in vitro* şartlarda bloke edebildiği için, C1D ve TAFI 48'in etkileşme ihtimali potensiyel bir önem arz etmektedir.

Bu çalışmada, C1D ve TAFI 48'in mayada, *in vitro* şartlarda ve memeli hücrelerinde spesifik olarak etkileşime girdiği gösterilmiştir. Bu etkileşimin çift sarmal DNA kırıklarının etkisiyle DNA-PK tarafından rDNA'dan transkripsiyonun bloke edilmesinin düzenlenmesinde potansiyel rolleri olduğu düşünülmektedir.

Anahtar Kelimeler: C1D, DNA-PK, çift sarmal DNA kırıkları, TAFI 48.

ACKNOWLEDGEMENT

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ABBREVIATIONS

BIR	break induced replication
bp	base pairs
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
DBD	LexA DNA binding domain
DSB	DNA double-strand break
ddH2O	deionized distilled water
DMEM	Dulbecco's modified Eagles' medium
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent Protein Kinase
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	diaminoethane tetra-acetic acid
EtBr	Ethidium Bromide
FCS	Fetal Calf Serum
GST	glutathione-S-transferase
HA	Hemagglutinin
HMG	High Mobility Group
HR	Homologous Recombination
HRP	Horse Radish Peroxide

ICR	Internal Control Region
Inr	Initiator Sequence
IPTG	Isopropylthio-b-D-galactosidase
IR	Ionizing Radiation
LB	Luria-Bertoni Media
MCS	Multiple Cloning Site
MMS	Methyl-methoxy Sulfide
NHEJ	Non-homologous End joining
NTA	Nitrilo-tri-acetic acid
OD	optical density
ONPG	o-nitophenyl b-D-galactopyranoside
PAGE	Polyacrylamide Gel Eletrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PMSF	Phenylmethyl-sulfonyl fluoride
Pol I	Polymerase I
PSE	Proximal Sequence Element
RNA	Ribonucleic Acid
RNAse	Ribonuclease
RPA	Replication Protein A
Rpm	rotation per minute
SD	synthetic dropout

SDS	Sodium Dodecyl Sulfate
SDSA	Synthesis-dependent Strand Annealing
SEM	Simple and Efficient Method
SL1	Selectivity Factor 1
snRNA	Small Nuclear RNA
SSA	single strand annealing
TAE	Tris-Acetic Acid-EDTA
TAF	TBP-associated factor
TB	transformation buffer
TBE	Tris-Boric Acid-EDTA
TBP	TATA Box-binding Protein
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with Tween
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
Tris	Tris (hydroxymethyl)-methylamine
UBF	Upstream Binding Factor
UCE	Upstream Control Element
UV	Ultraviolet
XRCC	x-ray cross complementing

INTRODUCTION

1. DNA DAMAGE

Cells are constantly under threat from the cytotoxic and mutagenic effects of DNA damaging agents. These agents can either be exogenous or formed within cells. Environmental DNA-damaging agents include UV light and ionizing radiation, as well as a variety of chemicals contained in foodstuff and cigarette smoke. Endogenous damaging agents include methylating species and the reactive oxygen species that arise during respiration.

These DNA damaging agents may lead to two general types of abnormalities that, if left unrepaired, will eventually lead to mutation, which is a permanent change in DNA sequence. The first class of mutations involve single base changes, which does not cause a major structural distortion on the DNA. The second class on the other hand, induces distortions on the structure of DNA, which may interfere with transcription and replication processes, with deleterious effects on the cell survival.

To ensure the genetic stability and cell survival, cells are equipped with mechanisms to repair these structural abnormalities. The first reaction in response to DNA damage is to arrest the cell cycle to provide enough time for the repair process. This event also prevents transmission of the damaged DNA to the next generations. Following the cell cycle arrest, repair process is initiated, using one of the many repair pathways differing according to the type of damage. However, if the damage is beyond repair, then cells choose to commit suicide (apoptosis) to prevent the cell being divided with the heavily mutated DNA. In general terms there are three categories of DNA repair. First one is excision repair, which includes both base and nucleotide excision repair. The incorrect or

damaged base is removed either as a base (base excision) or as an (oligo)nucleotide (nucleotide excision), the single-stranded gap resulting from the excision is filled by a polymerase using the information of the other strand. Second one is mismatch repair, which relies on the parental strand to correct misincorporation of nucleotides during replication and heteroduplex regions produced by genetic recombination. The third category is double-strand break repair and unlike the other two pathways, presence of homology regions is not necessarily required for this type of DNA repair.

1.1. DNA Double-Strand Break (DSB)

The DNA double-strand break (DSB) is one of the most dangerous lesions that can occur within the cell. The presence of an unrepaired DSB will trigger the DNA-damage response systems of a cell to arrest its progression through the cell cycle and, sometimes, to cause apoptotic cell death (Huang et al. 1996). If repaired improperly, they can lead to chromosome translocations and cancer.

DNA DSBs can arise by exogenous agents such as ionizing radiation (IR) and certain chemotherapeutic drugs, or as a consequence of some physiological events such as endogenously generated reactive oxygen species, mechanical stress on the chromosomes, or when DNA replication forks encounter DNA single-strand breaks or other type of lesions. DNA DSBs are also generated to initiate recombination between homologous chromosomes during meiosis and occur as intermediates during developmentally regulated rearrangements, such as V(D)J recombination and immunoglobulin class-switch recombination (Smider and Chu 1997).

There are two general types of DSB repair: homologous recombination (HR) and non-homologous end joining (NHEJ).

1.1.1. Homologous Recombination

There are several types of homologous repair: gene conversion, break-induced replication and single-strand annealing. After a double-strand break (DSB) is created, the ends are resected and one of the 3' single-stranded ends can invade an intact template. Strand invasion requires the participation of the Rad51p strand exchange protein and a number of associated proteins, including Rad52p, Rad54p and Tid1p. During meiosis, these recombination proteins are joined by a second strand-exchange protein, Dmc1p. Strand invasion is believed to establish a modified replication fork, in which both leading and lagging-strand DNA synthesis occurs. As new DNA synthesis proceeds, branch migration displaces the two newly synthesized strands. The four main outcomes of homologous repair can be summarized as follows: (a) If the replication fork encounters the other end of the DSB, an intermediate containing two Holliday junctions can be formed, allowing gene conversions to be resolved both with and without crossing-over. (b) If the strands are completely displaced or if the leading strand pairs with the second end of the DSB, a simple synthesis-dependent strand annealing (SDSA) will occur, producing gene conversions without crossing-over (Pâques and Haber 1999; Fishman-Lobell et al. 1992). (c) If the second end of the DSB fails to engage, replication can proceed all the way to the end of the chromosome (or until it encounters a converging replication fork). This process is known as break-induced replication (BIR) (Bosco and Haber 1998; Morrow et al. 1997). (d) If resection proceeds far enough to expose complementary strands of homologous

sequences flanking a DSB, repair can occur by single-strand annealing (SSA), leading to a deletion of all intervening sequences (Sugawara et al. 2000).

1.1.2. Non-Homologous End Joining (NHEJ)

DSB ends can be repaired by several nonhomologous repair mechanisms, in which the DNA ends are joined with little or no base pairing at the junction (Moore and Haber 1996; Jeggo 1998). NHEJ of two DNA ends does not require an undamaged partner and does not rely on extensive homologies between the two recombining ends. In this process, sometimes after limited degradation at the termini, the two ends are ligated together. Consequently, NHEJ is prone to error, and small sequence deletions are usually introduced.

End joining in yeast and mammals requires the same core set of proteins: the DNA end-binding proteins Ku70p and Ku80p, as well as DNA ligase IV and its associated Xrcc4 protein. Vertebrate cells also require DNA-PKcs, which together with the Ku70 and Ku80 forms the DNA-PK holoenzyme. DNA-PK is a nuclear serine/threonine kinase with a well-established role in NHEJ. Interestingly, no homologue has been identified in *S.cerevisiae* for DNA-PKcs. In mammals, the carboxy-terminal portion of Ku80 is essential for the activation of DNA-PK and this region is distinct from the one that interacts with Ku70 (Singleton et al. 1999). The requirement for this carboxy-terminal region in kinase activation is consistent with the absence of an analogous Ku80 carboxy-terminal tail in S.*cerevisiae,* which also lacks a homologous DNA-PKcs protein.

Although NHEJ has been considered the major pathway of DSB repair in mammalian cells, these two processes are in competition with each other. Very little is known in general about how cells choose which pathway to use. It has been suggested that

two DNA end-binding proteins, Rad52 and Ku compete for binding to DSBs and channel the repair of DSBs into HR and NHEJ, respectively (Van Dyck et al. 1999; Haber 1999).

1.2. Involvement of DNA-PK in NHEJ

The nuclear serine/threonine kinase, DNA-PK, is comprised of a catalytic subunit termed DNA-PKcs and two DNA binding componets, Ku70 and Ku80. The involvement of DNA-PK in DSB repair became evident from analyses involving a specific series of mutant rodent cell lines (Jackson and Jeggo 1995). Early studies in these mutant cells found them to be hypersensitive to irradiation (IR) and radiomimetic agents with little or no cross sensitivity to other types of DNA damaging agents and showed them to be defective in the repair of chromosomal DNA DSBs. Screening of these x-ray sensitive cell lines led to the identification of three distinct complementation groups, termed IR4, IR5, and IR7, with defects in both DSB repair and V(D)J recombination. Since efforts were undertaken to complement these rodent cell lines with human genes, the genes for the complementation groups were designated *XRCC* for x-ray cross complementing (Thompson and Jeggo 1995; Zdzienicka 1995). Later it was shown that, cells of IR5 and IR7 harboured inactivating mutations in the genes of Ku80 and DNA-PKcs, respectively, and that inactivation of Ku80 leads to a dramatic destabilization of both itself and Ku70 (Errami et al. 1996, 1998a, 1998b; Blunt et al. 1996; Danska et al. 1996; Araki et al. 1997; Singleton et al. 1997; Peterson et al. 1997; Fukumura et al. 1998; Priestley et al. 1998). It was therefore concluded that mutations in Ku80 and DNA-PKcs lead to IR sensitivity, that *XRCC5* and *XRCC7* encode Ku80 and DNA-PKcs respectively, and that DNA-PK is a crucial component of the mammalian DNA DSB repair apparatus (Priestley et al. 1998).

Although among these mutant cell lines, a mutation for Ku70 was not identified, later a group of cell line termed IR6 was established by knocking-out the Ku70 gene and was shown that these cells also carry the same phenotype as IR5 and IR7 and was defective in DNA-PK activity. Therefore, it was concluded that Ku70 is encoded by XRCC6 (Gu et al. 1997).

Although the precise molecular mechanism underlying the role of DNA-PK in DSB repair is not yet known, in the light of biochemical and genetics studies, some hypotheses have been proposed. The heterodimer of Ku80 and Ku70 binds with high affinity to DNA ends, suggesting an early role in damage recognition, processing or both. Ku binding probably protects the DNA ends from degradation until end joining is completed (Liang and Jasin 1996). In addition, Ku protein might play a role in juxtapositioning of two DNA ends since it can transfer between DNA fragments with complementary overhangs (Bliss and Lane 1997). On binding to the DNA ends, Ku may recruit DNA-PKcs. Synapsis of the two DNA ends might be mediated by two independent DNA binding sites on DNA-PK or by the association of two DNA-PK molecules (Chu 1997). The molecular architecture of DNA-PKcs suggests a structural role for the protein in the rejoining process. It has a potential DNA-binding groove and an enclosed cavity with three apertures through which single-stranded DNA could pass (Leuther et al. 1999). These findings are consistent with DNA-PKcs mediating the alignment of short stretches of single-stranded DNA prior to ligation.

DNA-PK has also an autophosphorylation activity (Chan and Lees-Miller 1996). It is hypothesized that this phosphorylation occurs *in trans*, so that the kinase assembled on one DNA end phosphorylates the other DNA-PK molecule assembled on the other DNA

end. Activity *in trans* would regulate the kinase activity so that processing of the DNA ends would occur only after synapsis of the two ends is accomplished (Chu 1997).

The autophosphorylation also causes the dissociation of the catalytic subunit from Ku (Chan and Lees-Miller 1996), and Ku acquires helicase activity (Cao et al. 1994; Tuteja et al. 1994). It is postulated that this helicase activity unwinds DNA ends *in vivo* so that exposed regions of microhomology can anneal by base pairing (Chu 1997). The unpaired DNA flaps could then be removed either by an exonuclease or a flap endonuclease. Gaps are then filled in by a DNA polymerase and the nicks sealed by ligase to complete the end-joining process (Figure 1, Chu 1997).

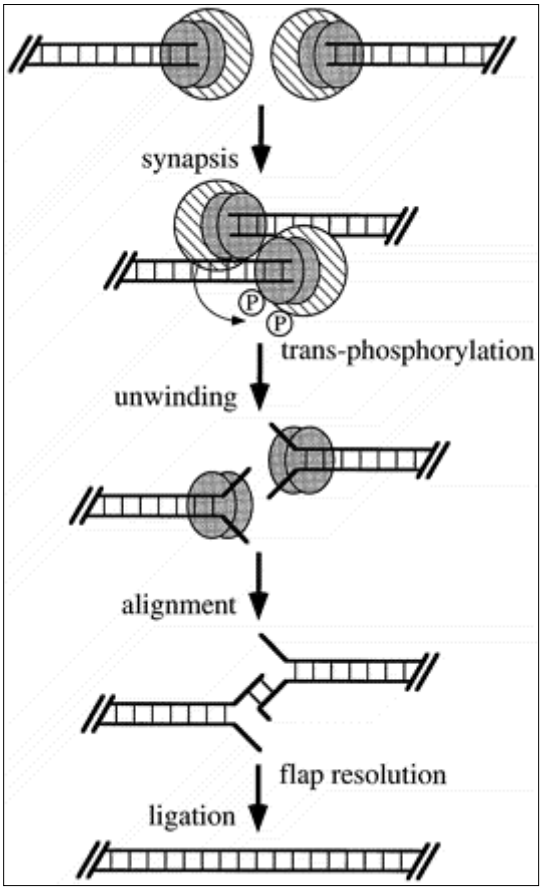

Figure 1.1: Model for the role of Ku and DNA-PKcs in nonhomologous end joining.

Once positioned at the DNA DSB, Ku and DNA-PKcs might then recruit other NHEJ factors essential for the repair process. It is possible that, as being a kinase, DNA-PK may also regulate the activity of the proteins in this repair complex by phosphorylation. In this regard, the plausible targets for DNA-PK include the single-stranded binding protein RPA (replication protein A), the DNA ligase IV cofactor Xrcc4, Ku, and DNA-PKcs itself (Karran 2000). However, DNA-PK has also been shown to phosphorylate many transcription factors, including the tumour suppressor p53 *in vitro*, which indicates that DNA-PK may also be involved in regulation of transcription. Indeed, DNA-PK has been demonstrated to repress Polymerase I transcription *in vitro* (Kuhn et al. 1995). All these findings suggest that DNA-PK may also regulate transcription at the sites of DNA damage and may be by repressing transcription and recombination, to allow enough time for the repair machinery to perform its function (Zhu et al. 1996).

2. **EUKARYOTIC TRANSCRIPTION**

In eukaryotes, transcription is carried out by three different RNA polymerases, RNA polymerase I, II, and III, each of which is dedicated to the transcription of different sets of genes. The genes in each class contain characteristic promoters, which often consist of two types of elements: the basal promoter elements and the modulator promoter elements. The basal promoter elements are sufficient to determine RNA polymerase specificity and direct low levels of transcription, whereas the modulator elements enhance or reduce the basal levels of transcription. None of the RNA Polymerases can recognize its target promoters directly. Instead, basal promoter elements are first recognized by specific transcription factors, which then recruit the correct RNA polymerase.

The RNA Polymerase I promoters, which direct transcription of the large rRNA genes, do not contain a TATA box (Reeder 1992). The human rRNA promoters are instead composed of a core element that overlaps the transcriptional start site and an upstream control element (UCE) located upstream of position –100 that stimulates transcription 10- to 100-fold.

RNA polymerase II promoters can be divided into two large classes: the mRNA promoters and the snRNA promoters. The mRNA promoters can in turn be divided into two classes depending on whether or not they contain a TATA box. The basal TATA-box containing mRNA promoters may consist of either the TATA box alone or the TATA box and an initiator sequence (Inr) that spans the transcriptional start site. The basal TATA-less mRNA promoters consist of just the Inr. Both TATA box and Inr appear to recruit the TBP-containing complex TFIID. TBP (TATA-box Binding Protein) on its own can, however, replace the TFIID complex to direct basal levels of transcription from TATA-containing promoters *in vitro*, suggesting that direct interactions exist between TBP and components of the basal transcription machinery.

The basal RNA polymerase II snRNA promoters consist of a proximal sequence element (PSE), which is also present in RNA polymerase III snRNA promoters. Transcription from these promoters also requires TBP, which is recruited by the PSE.

The RNA polymerase III promoters have been divided into three classes (Geiduschek and Kasavetis 1992). Classes 1 and 2 are gene-internal TATA-less promoters and consist of the internal control region (ICR) of 5S rRNA genes and the A and B boxes of tRNA and 7SL genes, Alu sequences, and viral genes such as the Ad2 VAI and VAII genes. Class 3 promoters are found in a number of vertebrate snRNA and cytoplasmic

RNA genes, including the U6, 7SK, hY1 and hY3, H1, and MRP/Th RNA genes (Hernandez 1992). They are located entirely upstream of the gene-coding sequences and are similar to the RNA polymerase II snRNA promoters except for the presence of a TATA box, which determines the RNA polymerase III specificity of the promoter. Transcription from both the TATA-less and TATA-containing RNA polymerase III promoters requires TBP, although in different complexes.

2.1. Regulation of RNA Polymerase I and Connection with DNA-PK

Human ribosomal RNA synthesis by RNA Polymerase I requires the upstream binding factor (UBF) and the promoter selectivity factor (SL1) for accurate initiation of transcription from the human ribosomal promoter (Learned et al. 1985; Learned et al. 1986; Bell et al. 1990). UBF binds in a sequence specific manner to both core and the UCE. In contrast, SL1 has no or little affinity for the rRNA promoter but binds cooperatively with UBF. SL1 consists of the TBP and three associated subunits, TAFI 110, TAFI 63 and TAFI 48 (Comai et al. 1992; Comai et al. 1994; Zomerdijk et al. 1994). TAFI 48 serves as a target for interaction with UBF and is required to form a transcriptionally active SL1 complex responsive to UBF *in vitro* (Beckmann et al. 1995). TAFI 48 also alters the ability of TBP to interact with TATA box elements, and the resulting complex fails to support transcription by RNA Polymerase II (Beckmann et al. 1995).

Transcription of ribosomal RNA genes by RNA polymerase I oscillates during the cell cycle, being maximal in S and G2 phases, repressed during mitosis, and gradually recovering during G1 progression. Silencing of cellular pre-rRNA synthesis during mitosis is caused by the inactivation of the Pol I-specific transcription initiation factor SL1 by

cdc2/cyclin B-mediated phosphorylation of TAFI 110 (Heix et al. 1998). Binding of SL1 to the core element of the ribosomal gene promoter is enhanced by the upstream binding factor (UBF), a member of the family of high mobility group (HMG) box proteins (Jantzen et al. 1990). Interestingly the chromatin associated high mobility group (HMG) proteins 1 and 2 have been shown to stimulate DNA-PK activity *in vitro* (Watanabe et al. 1994).

Mitotic phosphorylation has been demonstrated to impair the capability of SL1 to interact with UBF (Heix et al. 1998), indicating that phosphorylation of the Pol I-specific TBP-TAF complex is used as a molecular switch to prevent pre-initiation complex formation and rDNA transcription at mitosis.

DNA-PK was also found to be capable of repressing transcription by RNA Polymerase I *in vitro* (Kuhn et al. 1995; Labhart 1995). Transcriptional repression by DNA-PK is due to protein phosphorylation. DNA-PK inhibits transcription from both linear and circular templates, but the repression is more efficient on linear templates (Kuhn et al. 1995; Labhart 1995). Partial fractionation of the *in vitro* transcription system showed that a protein fraction containing transcription factor Rib1, the *Xenopus* equivalent of human SL1, mediated the repression of transcription by DNA-PK (Labhart 1995).

The effect of DNA-PK on Pol II and III has not been demonstrated so far. Moreover, the precise molecular mechanisms of Pol I repression by DNA-PK or whether DNA-PK is also capable of repressing Pol I transcription *in vivo* is not yet known.

3. DNA-PK AND THE NUCLEAR MATRIX

As summarised above, a wide range of functions have been proposed for DNA-PK many of which lack solid evidence. To date, although its involvement in DNA DSB repair

is well established, the molecular mechanisms that DNA-PK use while performing these functions are not yet clear. It is noteworthy to mention that DNA-PK is present at ~$1x10^5$ to $5x10^5$ molecules per human cell (up to 1% of HeLa cell nuclear protein), which is far in excess of the number of DNA DSBs generated by physiological doses of DNA damaging agents and its levels do not appear to be regulated strongly by DNA-damaging agents (Lee et al. 1997). This could be because of the need for an immediate response to DNA damage and by having huge amounts of DNA-PK, the damaged ends could be detected immediately without spending time for the new protein synthesis. However, the DNA is not naked in the cellular environment and the chromatin context has to be considered when proposing models for how DNA-PK binds to DNA and gets activated to perform its kinase function. It is possible that DNA-PK may need an accessory protein that would allow its access to DNA wrapped in chromatin, which may prevent the exposition of broken DNA ends. Indeed, during DNA-PK purification from human cells, a substantial proportion (40 %) of DNA-PK was found to be associated with the insoluble fraction of the nucleus, which constitutes the nuclear matrix (U. Yavuzer, unpublished data).

Nuclear matrix by definition is the structure that can be isolated from cells after removal of soluble proteins, histones and most of the DNA. A group of proteins however, stay tightly attached to DNA and cannot be removed even by treating with harsh denaturants. Accumulating evidence indicate the association of nuclear matrix with several nuclear metabolic processes including DNA replication, transcription, RNA splicing, topoisomerase activity, nucleotide excision repair, and DNA DSB repair (Berezney 1984; Cockerill and Garrard 1986; Nelson et al. 1986; Verheijen et al. 1988; Jackson 1991;

Kaufman and Shaper 1991; Yasui et al. 1991, 1994; Korte and Yasui 1993; Johnston and Bryant 1994; Koehler and Hanawalt 1996).

Interestingly, attempts towards identifying the molecular mechanisms that DNA-PK employ to perform its function revealed that a nuclear matrix protein C1D interacts specifically with the putative leucine zipper region of DNA-PKcs. This interaction is interesting in the sense that C1D is capable of activating DNA-PK in the absence of free DNA ends (Yavuzer et al. 1998). Moreover, the nuclear matrix protein C1D is phosphorylated very efficiently by DNA-PK and its mRNA and protein levels have been demonstrated to be induced upon γ-irradiation (Yavuzer et al. 1998), suggesting that C1D, therefore the nuclear matrix may play a role in DNA DSB-response pathway in connection with DNA-PK. It is possible that C1D targets DNA-PK to the nuclear matrix and matrix-associated DNA in response to DNA DSBs. Indeed, the *xrs*-5 cells deficient in one of the subunits of DNA-PK, Ku80, exhibit irregularly shaped nuclear envelope and altered nuclear matrix compared to their wild-type controls (Korte and Yasui 1993, Yasui et al. 1991).

Recently, in this department, the yeast two-hybrid system was employed (Dincer et al. unpublished) to identify the interacting proteins with C1D in order to reveal the biological functions of the nuclear matrix and DNA-PK. It was found that C1D interacts with a group of proteins where TAFI 48 subunit of SL1 is one of them (unpublished data). Given the connection between DNA-PK, Pol I and C1D, this interaction was thought to be potentially interesting as verification of the interaction might shed light on to some of the molecular mechanisms of how DNA-PK performs its function.

The aim of this project therefore, is to demonstrate that C1D and TAFI 48 interact in yeast, in mammalian cells and *in vitro* specifically with the hope that further characterization of this interaction may provide clues about how transcription from rDNA is regulated by DNA-PK in response to DNA damage.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. BACTERIAL STRAINS

The bacterial strains used in this study were DH5α and M15.

Table 4.1: List of the *E.coli* strains used during the course of this study

Strain	Genotype	Usage	Source
M15 (pREP4)	*F⁻, Nal^S, Str^S, rif^S, lac⁻, ara⁻, gal⁻, mtl⁻, recA⁺, uvr⁺*	Production of recombinant proteins with pQE vectors	Villarejo and Zabin 1974
DH5α	*F- supE44 hsdR17 recA1 gyrA96 endA1 thi-1 relA1 deoR lambda-*	a recA⁻ host for propagation and storage of plasmids	Cold Spring Harbour Labs.

4.2. GROWTH AND MAINTENANCE OF BACTERIA

Bacterial strains were stored in 50% glycerol at –70^{o}C for long-term storage. Overnight grown cultures to saturation were mixed with sterile glycerol with a ratio of 1:1, mixed to homogeneity, and stored at -70^{o}C until required. Bacteria was recovered by scraping a small amount of cells from the frozen stock with an inoculation loop and streaking onto a LB-agar plate (supplemented with the appropriate antibiotics).

Liquid culture of plasmid-carrying *E.coli* was performed in LB (5 g NaCl, 10 g Bacto-tryptone, 5 g yeast extract, 1 ml of 1 M NaOH, ddH_2O added to 1 L, sterilised by autoclaving) with appropriate antibiotic selection. Liquid cultures were constantly agitated in a rotary shaking incubator (~200 rpm). When supplementing LB-agar (LB medium with 1.5% agar) with antibiotic, the LB-agar was melted and allowed to cool to 55^{o}C before the

addition of antibiotic and pouring into 10 cm diameter plastic petri dish (Greiner Labortechnik). Plates were stored at 4^{o}C and air-dried prior to use.

The specialised components of all media were obtained from Difco Laboratories Ltd.

4.3. YEAST STRAINS

The yeast strain used in this study was L40.

Table 4.2: Features of the yeast strain L40

Strain	Genotype	Reporters	Transformation Markers
L40	***MATa,*** *his3Δ200, trp1-901, leu2-3, 112, ade2 LYS2::(lexA$_{op}$)$_4$-HIS, URA3::(lexA$_{op}$)$_8$-lacZ GAL4*	*HIS3, lacZ*	*Trp1, leu2*

L40 is a reporter host used when screening fusion libraries for proteins that interact with a target protein. This strain carries both LacZ and HIS3 reporters integrated to *URA3* and *LYS2* genes of the yeast genome (Hollenberg et al. 1995). The expression of the *LacZ* and *HIS3* coding sequences are driven, respectively by minimal *GAL1* and *HIS3* promoters fused to multimerized LexA binding sites.

4.4. GROWTH AND MAINTENANCE OF YEAST

Yeast strains were stored in 25% glycerol at –70^{o}C for long-term storage. Cultures grown in the appropriate Yc selective medium (1.2 g yeast nitrogen base without amino

acids, 5 g Ammonium sulfate, 10 g succinic acid, 6 g NaOH and 0.1 g of all the aminoacids apart from his, ura, trp and leu were dissolved in 1 L, and autoclaved) to saturation were mixed with sterile glycerol with a ratio of 3:1, mixed to homogeneity, and stored at -70^{o}C until required. Yeast cells were recovered by scraping a small amount of cells from the frozen stock with an inoculation loop and streaking them onto a YPAD (or appropriate Yc) agar plate.

Liquid cultures of yeast were performed in YPAD (10 g yeast extract, 20 g peptone, 0.1 g adenine, ddH_2O added to 900 ml, sterilised by autoclaving, cooled to ~55^{o}C and 100 ml of 20% filter-sterilised glucose added) or in the appropriate Yc medium. Liquid cultures were constantly agitated in a rotary shaking incubator (~200 rpm) at 30^{o}C.

4.5. MAMMALIAN CELLS

The only mammalian cell line used in this study was COS-7 (ATCC CRL-1651). This line was derived from the CV-1 cell line, African green monkey kidney origin, (ATCC CCL-70) by transformation with an origin defective mutant of SV40 that codes for wild-type T antigen.

4.6. OLIGONUCLEOTIDES

Primers used in this study were synthesised in the Beckman Oligo 1000M DNA Synthesiser (Beckman Instruments Inc. CA, USA) at Bilkent University, Department of Molecular Biology and Genetics (Ankara, Turkey).

Cloning Primers:

1. UY 21 TAFI 48 Full Length Forward PCR Primer:

5′ AGACGTCGACAAGGATCC ATG AGT GAT TTC AGT GAA GAA 3′
***Sal*I** ***Bam*HI**

2. UY 22 TAFI 48 Full Length Reverse PCR Primer:

5′ AGACCTCGAGTCTAGA TCA GAG TCT TGG ATT TAC AAT 3′
***Sal*I** ***Bam*HI**

3. UY 54 TAFI 48 Full Length Reverse PCR Primer:

5′ AGACTCTAGACT GAG TCT TGG ATT TAC AAT ACT 3′
***Bam*HI**

Table 4.3: Primer pairs used to amplify full-length open reading frame of TAFI 48

Primer Pairs	PCR Product Size	Annealing Temperature
UY21/UY22	1384 bp	61°C
UY21/UY54	1379 bp	58°C

4.7. STANDARD SOLUTIONS AND BUFFERS

TBE Buffer:

Working Solution:
45 mM Tris-borate
1 mM EDTA.

10x Stock Solution (1lt):
108 g Tris Base
55 g Boric Acid
40 ml 0.5 M EDTA, pH 8.0

TAE Buffer:

Working Solution
0.4 M Tris acetate
0.001 M EDTA

50x Stock Solution (1lt)
2 M Tris Base (242 g)
57.1 ml Glacial Acetic Acid
50 mM EDTA

Phosphate-buffered saline (PBS):

Working Solution, ~pH 7.3:	10x Stock Solution (1lt):
137 mM NaCl	80 g NaCl
2.7 mM KCl	2 g KCl
4.3 mM $Na_2HPO_4.7H_2O$	11.5 g $Na_2HPO_4.7H_2O$
1.4 mM KH_2PO_4	2 g KH_2PO_4

4x Tris-Cl/SDS, pH 6.8:
Tris base 18.2 g, SDS 0.4g, ddH_2O to 100 ml, adjusted to pH 8.8 with HCl.

4x Tris-Cl/SDS, pH 8.8:
Tris base 6.05 g, SDS 0.4 g, ddH_2O to 100 ml, adjusted to pH 6.8 with HCl.

10x SDS-PAGE Running Buffer:
Tris base 30.2 g, Glycine 144g, ddH_2O to 1L.

Working Solution:	
100 ml 10x stock	25 mM Tris
5 ml 20% SDS	192 mM Glycine
895 ml ddH_2O	0.1% SDS

2x SDS PAGE Loading Buffer:
25 ml 4xTris-Cl/SDS pH 6.8
20 ml glycerol
3 g SDS
2 ml 2-ME
1 mg bromophenol blue
Add ddH_20 to 100 ml

Transfer Buffer:
Working Solution (for proteins less than 150 kD):
25 mM Tris
192 mM Glycine
10% methanol

Working Solution (for bigger proteins):
50 mM Tris
384 mM Glycine
20% methanol
0.1% SDS

1x TE:
10 mM Tris, pH 8.0
1 mM EDTA, pH 8.0

4.8. RECOMBINANT DNA TECHNIQUES

4.8.1. Polymerase Chain Reaction

Polymerase chain reaction (PCR), a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA (Mullis and Faloona 1987), was performed to amplify the coding sequence of TAFI 48. PCR reactions were performed in 0.2 ml Thermowell^{TM} tubes (Corning Costar Corp.) using the GeneAmp PCR system 9600 (Perkin Elmer).

PCR reactions were carried out in a reaction volume of 50 µl containing 1-2 ng of template DNA, 5 µl 10x PCR buffer (Sigma), 5% DMSO (Sigma), 1.5 mM $MgCl_2$ (Sigma), 0.2 mM of each dNTP (MBI Fermentas), 50 pmol of each primer and 1 unit *Taq* DNA polymerase (Sigma).

The reaction was preheated to 94^{o}C for 5 minutes and then subjected to 30 cycles of denaturation (30 seconds at 95^{o}C), annealing (1 minute at 61^{o}C or 58^{o}C depending on Tm degrees of the primers), and elongation (90 seconds at 72^{o}C). At the end of the 30 cycles a final extension at 72^{o}C for 10 minutes was also applied. PCR products were assessed by agarose gel electrophoresis and EtBr staining.

4.8.2. Phenol-chloroform extraction and ethanol precipitation of PCR products

This method was routinely used to purify and concentrate DNA preparations. The volume of the DNA solution was brought to 100 µl with ddH_2O and an equal volume of phenol was added on the solution to remove any protein contaminants. The mixture was vortexed vigorously and centrifuged at 13000 rpm for 2 minutes. The upper aqueous layer was removed, transferred into a new tube and an equal volume of chloroform was added to

remove any residual phenol. Once again the mixture was vortexed and centrifuged at 13000 rpm for 2 minutes. The upper aqueous layer was removed and transferred into a new tube. To the estimated volume of the solution, 1/10 volume of 3 M NaAc pH 5.2, and 3 volumes of ice-cold absolute ethanol was added. The solution was then frozen at –70^{o}C for 10-30 minutes, followed by centrifugation at 13000 rpm for 20 minutes at 4^{o}C. The supernatant was removed and the pelleted DNA was washed with 70% ethanol to remove salts and small organic molecules and dried at 37^{o}C. Finally, DNA was resuspended in ddH_2O at a concentration suitable for further experimentation and stored at –20^{o}C.

4.8.3. Purification of DNA fragments by Agarose Gel Electrophoresis

The samples were loaded into a single well of a 0.8%-1% low melting temperature agarose gel containing 1μg/ml EtBr. After electrophoresis, the DNA bands were visualised under UV illumination and the desired bands were excised from the agarose gel with a surgical blade (Aesculap Surgical Industries). Agarose slices were suspended in about 300 μl of a buffer containing 10 mM Tris-HCl pH 8, 1 mM EDTA, and 100 mM NaCl. The gel slices were melted down by heating at 67^{o}C for 20-30 minutes and while it was still at 67^{o}C, an equal volume of phenol was added to the melted gel. The mixture was vortexed well and centrifuged for 15 minutes in a microfuge at 13000 rpm.

The melted gel material forms a thick precipitate at the interface. The aqueous phase was removed and was extracted once again with phenol, twice with an equal volume of chloroform and optionally once with an equal volume of water-saturated ether. Each extraction was done by vortexing for a few seconds and between extractions, aqueous phase was recovered by centrifugation in a microfuge for a few seconds at the maximum

speed. If used, residual ether after the last extraction was removed by heating the sample for 3 min at 67^{o}C.

The DNA was precipitated with 3 volumes of absolute ethanol for 30 min to overnight at –70^{o}C. The DNA was pelleted by centrifugation at the maximum speed for 30 min at 4^{o}C, followed by washing once with 0.5 ml of 70% ethanol. The residual ethanol was removed by drying the pellet at 37^{o}C for 2 minutes. The DNA pellet was resuspended in appropriate volume of ddH_2O**.**

4.8.4. Restriction Enzyme Digestion of DNA

Depending on the amount of DNA (1-5 μg), restriction enzyme digests of DNA was carried out in a total volume of 20-50 μl with 10-50 units of restriction enzyme, volume of which was never greater than 1/10th of the total reaction volume. Appropriate buffers and incubation conditions were used for each digest as recommended by the manufacturer.

4.8.5. DNA Ligation

DNA fragments were ligated into plasmid vectors as described in Molecular Cloning (Maniatis et al. 1982). Prior to ligation, vector and insert concentrations were checked by agarose gel electrophoresis. For undirectional cloning, vector: insert ratio was set to 1:3 in molar terms and for directional cloning vector: insert ratio was kept close to 1 or slightly bigger than 1 in the ligation reactions. Ligations were performed in 15 μl reaction volumes containing 0.1-0.2 μg of plasmid DNA and the corresponding amount of insert in the presence of 4 Weiss units of T4 DNA ligase, 1mM ATP and standard ligation

buffer supplied by the manufacturer. The reaction was kept at room temperature for 4 hours or at 16^{o}C overnight.

4.8.6. Plasmids

The plasmids used in this study to clone the full-length open reading frame of TAFI 48 are: pACT2, pQE30, T7-myc-pLink, pCMV 5'2N3T, pcDNA3.1/Myc-HisB and pBlueBacHis2. The available maps are presented in figures 4.1, 4.2, 4.3 and 4.4.

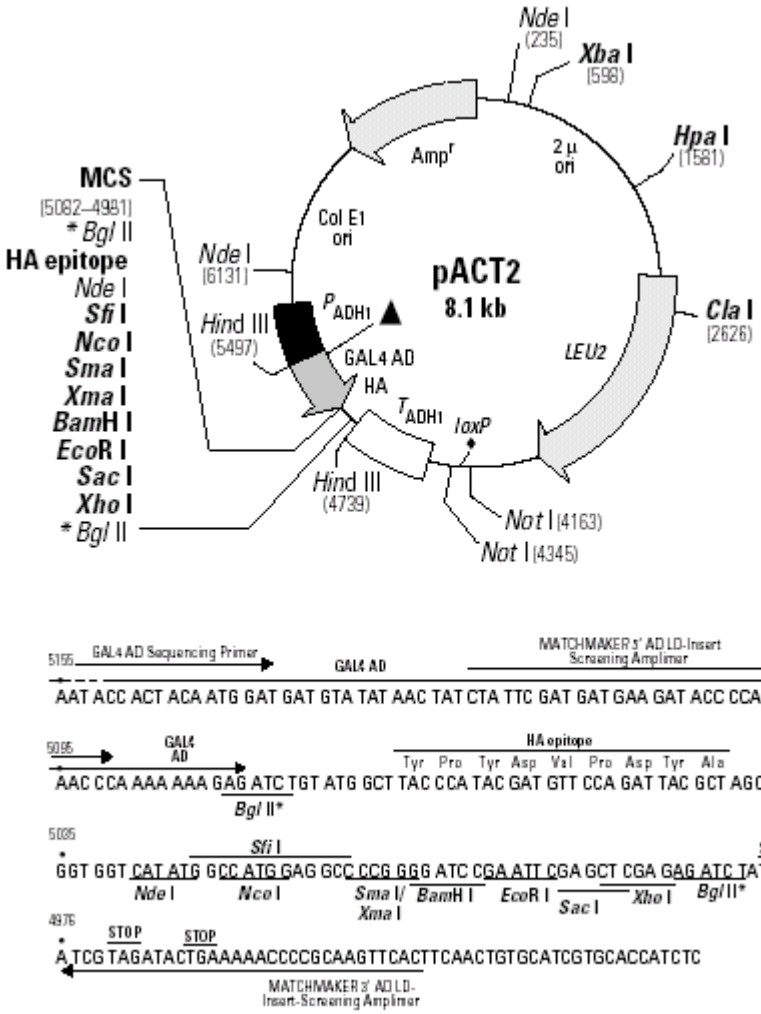

Figure 4.1: Restriction Map and Multiple Cloning Site (MCS) of pACT2

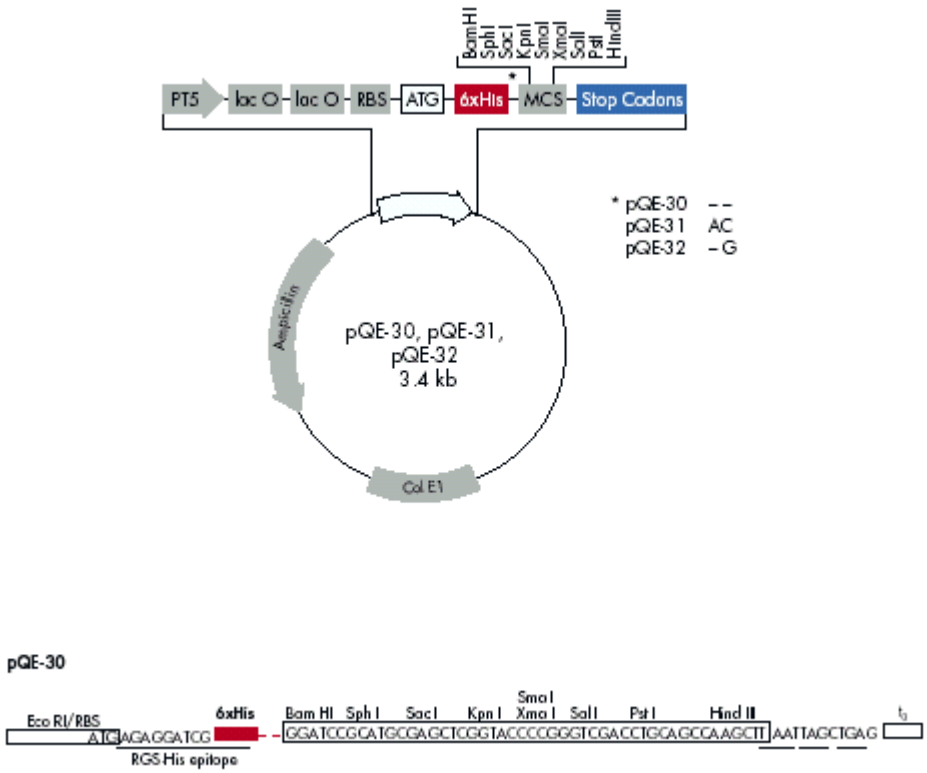

Figure 4.2: Map of pQE30 vector and its MCS

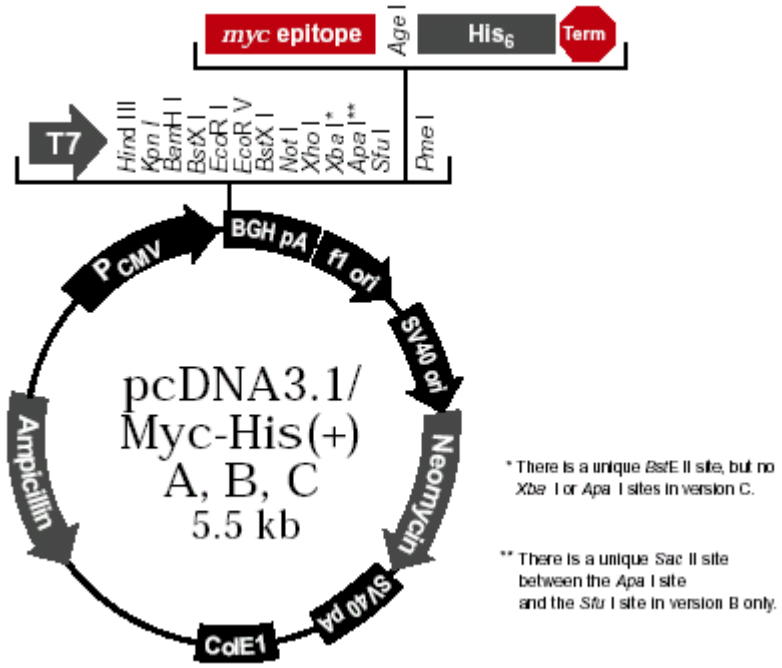

Figure 4.3: Map of pcDNA3.1/Myc-His vectors

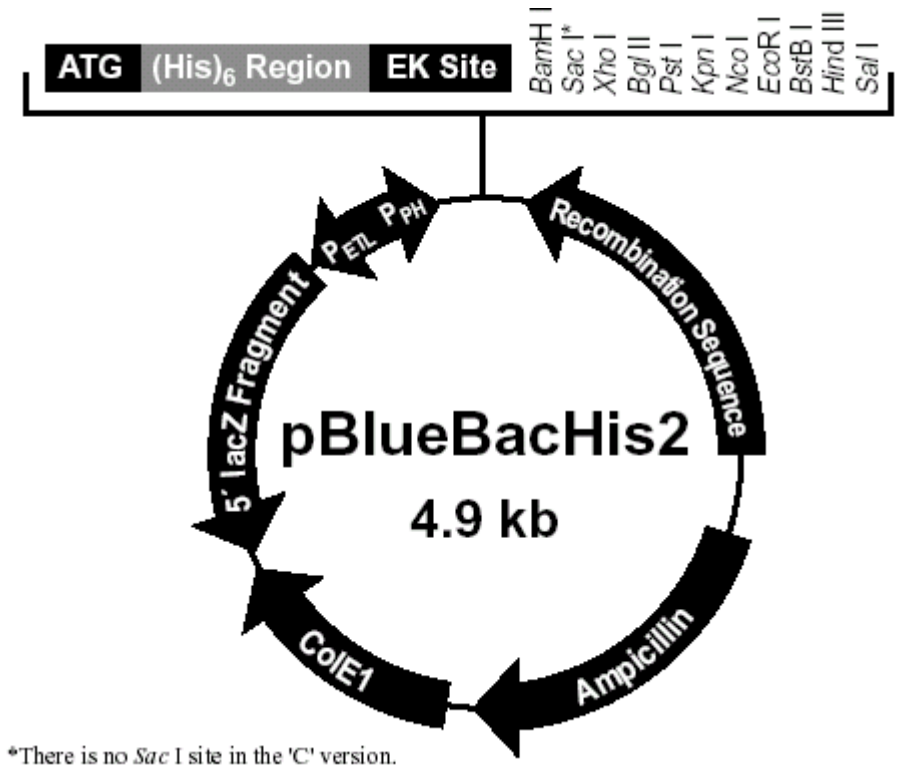

Figure 4.4: Map of pBlueBacHis2

4.8.7. Recombinant Expression Constructs

Constructs required for yeast, bacterial, mammalian and baculovirus expression, *in vitro* transcription and translation were prepared as follows. Inserts and plasmids were digested with appropriate restriction enzymes, were run on agarose gel for concentration determination and were ligated as described in the relevant section. Half of the ligation product was transformed into bacteria and recombinant plasmids were isolated (see sections 4.9. and 4.10.).

Table 4.3: List of the constructs prepared during the course of this study

Vector Digest	Insert Digest	Construct	Usage
pACT2 (*Xho*I)	UY21/22 (PCR pr.) (*Sal*I/*Xho*I)	TAFI 48-pACT2	Yeast expression
pQE30 (*Bam*HI/*Sal*I)	TAFI 48-pACT2 (*Bam*HI/*Xho*I)	TAFI 48-pQE30	Bacterial protein expression and purification
T7-myc-pLink (*Bam*HI/*Xho*I)	TAFI 48-pACT2 (*Bam*HI/*Xho*I)	TAFI 48-T7-myc-pLink	*in vitro* transcription and translation
pCMV 5'2N3T (*Sal*I)	UY21/22 (PCR pr.) (*Sal*I/*Xho*I)	TAFI 48-pCMV	Mammalian expression
pcDNA3.1/Myc-HisB (*Bam*HI/*Xba*I)	UY21/54 (PCR pr) (*Bam*HI/*Xba*I)	TAFI 48-pcDNA3.1	Mammalian expression
pBlueBacHis2 (*Bgl*II/*Sal*I)	TAFI 48-T7-myc-pLink (*Bam*HI/*Xho*I	TAFI 48-pBlueBacHis2	Baculovirus expression

4.9. PREPARATION OF COMPETENT CELLS AND TRANSFORMATION OF *E.COLI*

4.9.1. $CaCl_2$ Method

A single colony of an appropriate *E.Coli* strain was inoculated in 5 ml of LB (containing the appropriate antibiotics) and grown overnight at 37°C. The starter culture was diluted to an $O.D_{600}$ of 0.2-0.3 in 50 ml of LB (containing the appropriate antibiotics) and was grown further until the $O.D_{600}$ reached to 0.6-0.7. The culture was cooled on ice for 10 minutes and the cells were pelleted by centrifugation at 4000 rpm for 10 minutes at 4°C. The pellet was resuspended in 40 ml of ice cold filter-sterilised 100 mM $CaCl_2$ and incubated on ice for 1 hour. The cells were then pelleted as above and resuspended in 2 ml of ice cold sterile 100 mM $CaCl_2$. The competent cells prepared by this way can be kept on ice till transformation up to 24 hours (Cohen et al. 1972)

4.9.2. Simple and Efficient Method (SEM)

A single colony of appropriate *E.coli* strain was inoculated into 15 ml of LB (containing the appropriate antibiotics) and grown overnight at 37°C. The starter culture was diluted to an $O.D_{600}$ of 0.2-0.3 in 250 ml of SOB medium (2% Bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, sterilised by autoclaving) and grown to an OD_{600} of 0.6 at 18°C with shaking at 200-250 rpm. The culture was chilled on ice for 10 minutes, centrifuged at 2500 g for 10 minutes at 4°C. The pellet was then resuspended in 80 ml of ice-cold TB (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7, sterilised by filtration through 0.45 μm filter, stored at 4°C), and incubated on ice for 10 minutes. The mixture was pelleted as above, resuspended

gently in 20 ml of TB. DMSO was added to a final concentration of 7%, mixed gently and incubated on ice for 10 minutes. Aliquots of this mixture were then immediately chilled in liquid nitrogen, and stored at –70^{o}C up to 3 months without loss of transformation efficiency (Inoue et al. 1990).

4.9.3. Transformation of *E.Coli*

For transformation using competent cells prepared by $CaCl_2$ method, DNA (less than 100 ng) was added to 200 µl of competent cells in a microfuge tube, and incubated on ice for 30 minutes. The cells were then exposed to a 90 seconds heat shock at 42^{o}C, chilled on ice for 1-2 minutes, and 800 µl of LB was added and followed by incubation at 37^{o}C for one hour. The culture was then pelleted, resuspended in 100 µl of LB, and was spread on LB-agar plates supplemented with appropriate antibiotics (50 µg/ml ampicillin and/or 25 µg/ml kanamaycin). The plates were left for drying, then inverted and incubated overnight at 37^{o}C (Cohen et al. 1972).

For transformation with supercompetent cells, an aliquot of frozen competent cells was thawed on ice. 200 µl of the cells was mixed with plasmid DNA (less than 100 ng) in a 15 ml round bottom tube (Greiner Labortechnik), and incubated on ice for 30 minutes. The cells were then exposed to a 30 seconds heat shock at 42^{o}C, chilled on ice for 1-2 minutes, 800 µl of SOC (SOB with 20 mM glucose) was added and the cells were grown at 37^{o}C for one hour with vigorous shaking at 250 rpm. The culture was then pelleted, resuspended in 100 µl of SOC, and was spread on LB-agar plates as described above (Inoue et al. 1990).

4.10. PLASMID DNA ISOLATION

4.10.1. Small Scale Plasmid Isolation

Small-scale preparation of plasmid DNA was performed by standard methods based on NaOH/SDS cell lysis and potassium acetate precipitation of cellular debris (Maniatis et al. 1982).

Cells were harvested by centrifugation (13,000 rpm, 3 minutes, at room temperature) from a 1.5 ml overnight culture of bacteria carrying the plasmid of interest. Following resuspension of the bacterial pellet in 100 µl of solution I (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA), 200 µl of freshly prepared solution II (200 mM NaOH, 1% SDS) was added and cells were lysed by gently inverting the tube a few times. Bacterial chromosomal DNA and proteins were precipitated by the addition of 150 µl of solution III (3 M potassium acetate, 11.5% glacial acetic acid). An equal volume of phenol: chloroform (1:1) mixture was added and the mixture was vortexed well. The phases were separated by centrifugation at 13,000 rpm for 5 minutes at room temperature and upper aqueous layer was transferred into a new tube. The DNA was precipitated from the aqueous phase by addition of 1 ml of cold absolute ethanol and the DNA was pelleted by centrifugation at 13000 rpm for 20 minutes at 4^{o}C. After washing once with 70% ethanol, the pellet was dried and resuspended in 30 µl of ddH_2O with 10 µg/ml DNase-free RNase A (Roche Molecular Biochemicals) to remove any RNA contaminant.

4.10.2. Medium Scale Isolation (Midi-Preparation)

Cells were grown in 50-100 ml of LB (containing the appropriate antibiotic) overnight to saturation and plasmid DNA was isolated by using Nucleobond AX 100 (Macherey-Nagel), according to the manufacturer's instructions.

4.10.3. Spectrophotometric Quantification of DNA

The amount and the purity of DNA samples were determined by reading the absorbance of samples at 260 nm and 280 nm in a spectrophotometer (Beckman Instruments Inc., CA; USA). Nucleic acid samples displaying OD_{260} /OD_{280} ratio in the range of 1.8 to 2.0 are regarded as highly pure. A value of OD_{260}=1.0 corresponds to a concentration of approximately 50 μg/μl for double stranded DNA, 40 μg/ml for single stranded DNA and 20 μg/μl for oligonucleotides (Maniatis et al. 1982).

4.11. YEAST TECHNIQUES

4.11.1. Small Scale Yeast Transformation

A single colony of L40 was inoculated in 10 ml of YPAD and grown overnight at 30^{o}C with shaking at 200 rpm. The starter culture was diluted to an $O.D_{600}$ of 0.2-0.3 in 50 ml of YPAD medium and was grown further until the OD_{600} reached to 0.6-0.7. All subsequent steps were carried at room temperature. Yeast cells were pelleted at 2500 rpm for 10 minutes. After resuspending the pellet in 40 ml 1x TE, cells were repelleted and resuspended in 2 ml of LiAc/TE (100 mM LiAc in 0.5x TE), and incubated at room temperature for 10 minutes. Meanwhile 0.1 μg of DNA was dissolved in 8 μl of ddH_2O

and 10 μl of salmon sperm DNA was added. 175 μl of competent yeast cells was dispensed to DNA tubes, mixed by flicking. 700 μl of 100mM LiAc/ 40% PEG-3350/1x TE was added and incubated at 30°C for 30 minutes on a rotating wheel. Subsequently, 88 μl of DMSO was added, followed by a heat shock at 42°C for 7 minutes. Cells were then pelleted at full speed for 10 seconds, supernatant aspirated, and the pellet was resuspended in 1.0 ml of 1x TE. Cells were pelleted once more as above, resuspended in 100 μl of 1x TE, and were plated on the appropriate selective medium (Ito et al. 1983, Gietz et al. 1992).

4.11.2. Liquid Culture Assay Using ONPG as Substrate

Liquid yeast cultures were assayed for β-galactosidase activity to verify and quantify the interaction between two proteins of interest. L40 cells were cotransformed with the plasmid expressing TAFI 48 in fusion with the activation domain of GAL4 (TAFI 48-pACT2) and each of the different baits expressing Lamin, Daughterless and C1D in fusion with a DNA binding domain (LexA DNA-binding domain (DBD) alone, DBD-Lamin, DBD-Daughterless, or DBD-C1D). 3 colonies were picked from each transformation plate and were grown in 4 ml of the appropriate selective liquid medium for 2 days. 2 ml of the saturated culture was transferred into 8 ml of YPAD. The cultures were incubated at 30°C for 3-5 hrs with shaking (230-250 rpm) until the cells grew to the mid-log phase ($O.D_{600}$ = 0.5-0.8). The exact value of $O.D_{600}$ was recorded when harvesting the cells. 1.5 ml of culture was placed into each of three 1.5-ml microcentrifuge tubes. Cells were collected by centrifugation at 13000 rpm for 30 sec. After the supernatants were removed, the cells were resuspended in 1.5 ml of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7). Cells were centrifuged again and

supernatants were removed. Each pellet was resuspended in 300 μl of Z buffer so that the concentration factor was 5 fold. 0.1 ml of the cell suspension was transferred into a fresh microcentrifuge tube. Cells were lysed by freezing in liquid nitrogen for 30 seconds, followed by thawing in a 37^{o}C water bath. The freeze/thaw cycle was repeated for two more times to ensure that the cells had broken open. A blank tube with 100 μl of Z buffer was set up. 0.7 ml of Z buffer (+ β-mercaptoethanol) was added to the reaction and blank tubes. The tubes were placed in a 30^{o}C heat block. 160 μl of 4 mg/ml ONPG (Sigma) (prepared in Z buffer) was added to the reaction and blank tubes and the elapsed time until the yellow colour developed was recorded. When the colour developed, 0.4 ml of 1M Na_2CO_3 was added to the tubes to stop the reaction. The reaction tubes were centrifuged for 10 minutes at 13000 rpm to pellet the cell debris and the supernatants were transferred into clean cuvettes. The spectrophotometer was calibrated against the blank tube at A_{420} and the $O.D_{420}$ of the samples relative to the blank were measured. Finally, β-galactosidase units was calculated according to the following formula:

β-Galactosidase units $=1000 \times OD_{420} / (t \times V \times OD_{600})$

Where: t = elapsed time (in min) of incubation

V = 0.1 ml x concentration factor

1 unit of β-galactosidase is defined as the amount that hydrolyzes 1 μmol of ONPG to *o*-nitrophenol and D-galactose per min per cell (Miller 1972).

4.12. CELL CULTURE TECHNIQUES

4.12.1. Thawing a Frozen Cell Line

Frozen cell line stock in cryotube was transferred from liquid nitrogen into ice and then the frozen cells were thawed at 37^{o}C in less than 2 minutes. After thawing, cells were transferred into a 15 ml falcon tube and 10 ml of warmed fresh culture medium (DMEM supplemented with 10% FCS and 1% Penicillin/Streptomycin, all purchased from Biochrom) was added dropwise. Cells were collected by centrifugation at 1500 rpm for 5 minutes at 4^{o}C and the medium was removed by aspiration without disturbing the cell pellet. The pellet was then resuspended in 5 ml of fresh culture media and transferred into a 25 cm^2 culture flask (Greiner Labortechnik). The culture flask was incubated at 37^{o}C in a humidified atmosphere with 5% CO_2 for cell growth (Doyle and Griffiths 1997).

4.12.2. Sub-Culturing of Monolayer Cells

When reached to 80% confluence, cells were sub-cultured into new flasks. The medium was aspirated and monolayer cells were rinsed quickly with a volume of 0.25% trypsin/0.03% EDTA solution (Biochrom) just enough to cover the surface. The solution was removed and an additional amount of trypsin-EDTA solution was added. After incubation at 37^{o}C for 30 seconds, the trysin-EDTA solution was removed and the flask was allowed to sit at 37^{o}C until the cells detached. 2-10 ml (depending on the size of the culture flask) of culture media was added and any cell remaining attached was suspended by gently pipetting. The medium was transferred to a fresh 15 ml falcon tube. Following centrifugation at 1500 rpm for 5 minutes at 4^{o}C, the medium was removed by aspiration without disturbing the cell pellet. Then the pellet was resuspended in 5 ml of fresh culture

media and was dispensed into new culture flasks at the desired sub-culturing ratio. Additional fresh culture medium was added and the flasks were incubated at 37^{o}C in a humidified atmosphere with 5% CO_2 (Doyle and Griffiths 1997).

4.12.3. Cryopreservation

As in sub-culturing the cells were collected but instead of the culture medium, cells were resuspended in adequate amount of freezing medium (DMEM supplemented with 5% FCS and 10% DMSO) to give a cell density of 5 x 10^6 cells /ml, dispensed into cryotubes and were frozen overnight at –80^{o}C from where transferred into liquid nitrogen (Doyle and Griffiths 1997).

4.12.4. Transient Transfections

All transfections were performed with the FuGENE6 (Roche Molecular Biochemicals) transfection reagent according to the manufacturer's instructions. One-day before transfection, 2 x 10^5 cells in 4 ml total media were plated into a 60 mm culture dish (Greiner Labortechnik). On the day of transfection, cells should be 50-80% confluent and most cell lines plated at a density of 2 x 10^5 cells/ 4 ml in 60 mm culture dishes will achieve this confluency after overnight incubation at 37^{o}C with 5% CO_2.

4.13. GEL ELECTROPHORESIS

4.13.1. Agarose Gel Electrophoresis

DNA fragments were separated by gel electrophoresis using agarose at concentrations of 0.8-2.0% w/v in 1x TBE buffer. Samples were mixed with a fifth volume

of loading dye (8% deionised formamide, 1x TBE and 0.1% bromophenol blue) and the samples were loaded onto the gel. Electrophoresis was performed by running the gel at 100V. At the mid of the run, the gel was soaked briefly in 1x TBE containing 0.1% EtBr, and then was run further to completion. Gels were visualised by illuminating with UV light on a transilluminator at 302nm. The gel photos were captured with BioRad Multi-Analyst Software running on a PC and a hardcopy was produced by Lexmark Optra laser printer.

4.13.2. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoretic separation of proteins under denaturing conditions was performed by following the methods described in Current Protocols (Ausubel 1987). Resolving gels were made up to the required concentration, 10-15%, from a 40% acrylamide stock solution (38% acrylamide, 2% bisacrylamide, Severn laboratories). The electrophoresis apparatus (Mini-Protean 3, BioRad) was assembled according to manufacturer's instructions. Acrylamide stock solution (to the desired gel percentage) and 4x Tris-Cl/SDS pH 8.8 were mixed in ddH_2O and 10% ammonium persulphate and TEMED (30 µl and 10 µl, respectively for a 5 ml solution) were added to assist polymerisation. The mixture was then poured into the gap between the glass plates so that sufficient space is left for the stacking gel and water saturated isobutanol was layered immediately onto the top of the gel. After the polymerisation, the isobutanol layer was removed, washed with water and the stacking gel (0.49 ml of acrylamide stock solution, 3.225 ml ddH_2O, 1.25 ml 4x Tris-Cl/SDS pH 6.8, 30µl of 10% ammonium persulphate, 5 µl of TEMED) was poured onto the top of the resolving gel. The comb was immediately inserted into the stacking gel

avoiding the formation of bubbles. After the stacking gel polymerised, the comb was carefully removed and the wells were rinsed with the running buffer to remove any unpolymerised acrylamide. The samples were mixed with appropriate volume of 2x SDS loading buffer, boiled at 100^{o}C for 5 minutes to denature proteins, centrifuged at full speed for 5 minutes, and then loaded on to gel. Gels were run at 70V until the samples left the stacking gel. As the samples passed to the resolving gel the voltage was raised to 100V. After the electrophoresis, to detect the proteins, gel was carefully removed and stained in Coomassie Brilliant Blue (0.25% Coomassie Brilliant Blue G250, 45% methanol, 10% acetic acid) for 20 minutes, and destained in a solution of 30% methanol, 10% acetic acid, and 60% deionised distilled water for 1-4 hours. Alternatively gels were subjected to electrophoresis for the purpose of immunoblotting.

4.14. BIOCHEMICAL TECHNIQUES

4.14.1. Immunological Detection of Immobilized Proteins (Western Blotting)

4.14.1.1 Transfer of Proteins onto Membranes

Following SDS-PAGE, proteins were transferred from the gel to a nitrocellulose membrane (Schleicher & Schuell) using Mini Trans- Blot Cell (BioRad). Two sheets of 3 MM Whatman paper (Whatman International Ltd.) and one sheet of nitrocellulose membrane were cut to the dimensions of the gel to prepare the transfer stack. Nitrocellulose membrane was wetted in ddH_2O by capillary action. The gel, the membrane, Whatman papers and fiber pads were soaked in transfer buffer (25 mM Tris, 192 mM Glycine, 10% methanol or 50 mM Tris, 384 mM Glycine, 20% methanol and 0.1% SDS) for 15 minutes. Then the transfer stack was assembled in the following order

starting from the side that would face the cathode: fiber pad, one sheet of Whatman paper, the gel, the nitrocellulose membrane, one sheet of Whatman paper and finally the second fiber pad. While preparing the transfer stack, formation of any air bubbles between the layers was avoided. The transfer stack was then placed into the tank filled with transfer buffer and transfer was performed at 100V for 1 hour for proteins with molecular weights smaller than 150 kD and at 40V for 2 hours for bigger proteins.

4.14.1.2. Immunological Detection of Immobilized Proteins

After the transfer has been completed, the membrane was washed twice with TBS-T (TBS + 0.5% Tween-20), and incubated in 50 ml of blocking solution (10% non-fat dry milk in TBS-T) with continuous shaking for two hours at room temperature or overnight at 4^{o}C. After blocking, the membrane was washed four times for 10 minutes each with TBS-T. The membrane was then incubated with the solution containing the primary antibody (diluted in 1% non-fat dry milk in TBS-T) for 1 hour. The membrane treated with the primary antibody was washed four times for 10 minutes each with TBS-T. The membrane was then treated with the HRP (horse reddish peroxidase) conjugated secondary antibody (diluted in 1% non-fat dry milk in TBS-T) for 1 hour and was washed as described before.

The immunodetection with HRP conjugated secondary antibody was carried out by using Amersham LIFE SCIENCE ECL Western blotting detection reagents according to the manufacturer's instructions. The bands were visualised by exposing the membrane on photograph films.

4.14.1.3. Striping and Reprobing Membranes

This method was used to remove the primary and secondary antibodies completely from membranes. The membrane was submerged in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-Cl pH 6.7) and the membrane was incubated at 65^{o}C for 30 minutes with occasional agitation. The membrane was then washed four times for 10 minutes with large volumes of TBS-T at room temperature and all steps described above were applied for the immunodetection.

4.14.1.4. Peptide Neutralisation of Primary Antibody

For peptide neutralisation, primary antibody was combined with a five-fold (by weight) excess of blocking peptide in a small amount of PBS. The mixture was incubated for 2 hours at room temperature. Following neutralisation, antibody/peptide mixture was diluted in 1% non-fat dry milk in TBS-T and the protocol for immunological detection was performed as described above.

4.14.2. Immunoprecipitation

After thirty hours following transfection, the medium was aspirated and monolayer cells were rinsed twice with PBS at room temperature. All the following steps were carried out at 4^{o}C using ice-cold buffers. 1.5 ml of PBS + 50 mM EDTA was added on monolayer cells and cells were incubated on a shaker for 10 minutes. After incubation, cells were scraped with a rubber scraper and were transferred into a microcentrifuge tube . Cells were collected by centrifugation at 2000 rpm for 5 minutes. The supernatant was removed; cells were resuspended in 0.1 ml of Lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris pH

8.0, 0.5% NP-40, 10% glycerol, 1x Complete™ protease inhibitors) and were incubated on a rotating wheel for 30 minutes. Following incubation cell lysates were spun down at 10,000g for 10 minutes to remove the insoluble cell debris. The supernatant, which is the total cell lysate, was removed into a new tube and was incubated with the appropriate antibody for 2 hours on a rotating wheel. Meanwhile 20 µl of resuspended volume of the appropriate agarose conjugate (10 µl of Protein A-agarose + 10 µl of Protein G-agarose) was washed three times with 0.5 ml of Lysis Buffer (10 minutes washing followed by 5 minutes centrifugation at 2500 rpm to remove supernatant). After 2 hours incubation, cell lysate/antibody mix was added onto equilibrated beads and was incubated for further 2 hours. The beads were collected at the end of the incubation by centrifugation at 2500 rpm for 5 minutes and the supernatant was removed. Then the beads were washed four times with 0.5 ml of Lysis buffer as described before. After the last wash, supernatant was discarded and the beads were resuspended in 20 µl of 2x SDS Loading Buffer. The samples were boiled for 5 minutes, centrifuged at full speed for 5 minutes and then loaded on to SDS PAGE gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane and the immunological detection of the immobilized proteins was performed.

4.14.3. Protein Purification of Glutathione-S-Transferase Tagged C1D Protein

The pGEX vectors are designed so that foreign polypeptides can be expressed in *E.coli* in a form that allows them to be purified rapidly under nondenaturing conditions. Foreign polypeptides are expressed as fusions to the C terminus of glutathione-S-transferase (GST), a common 26 kD cytoplasmic protein of eukaryotes. The fusion protein typically remain soluble within the bacteria and can be purified from lysed cells because of

the affinity of the GST moiety for glutathione immobilised on agarose or sepharose beads. Recovery of the fusion protein is by elution with free reduced glutathione at reduced pH (Ausubel 1987).

A single colony of M15 strain of *E.Coli* transformed with pGEXTK2-C1D construct was inoculated in 5 ml of LB (supplemented by 50 µl/mg ampicillin and 25 µl/mg kanamaycin) and grown overnight at 37^{o}C. The starter culture was diluted to an $O.D_{600}$ of 0.2 in 50 ml of LB (containing the appropriate antibiotics) and was grown at 30^{o}C till its $O.D_{600}$ reached to 0.55. IPTG (MBI Fermentas) was added to a final concentration of 0.15 mM and cells were grown further for 3 hours at 30^{o}C. Cells were then harvested at 4000 rpm for 20 minutes at 4^{o}C, and then resuspended in 1 ml TBS-PMSF (1% Triton, 1mM PMSF, BSA 10 µg/ml, 1x Complete^{TM} protease inhibitors diluted in TBS). Cells were then sonicated on ice giving 10 seconds bursts at 300 W by the aid of UP 50 Ultrasonic Processor (UniEquip), and were centrifuged at 8000 rpm for 30 minutes at 4^{o}C. Meanwhile, 30 µl of glutathione-sepharose-4B beads (Pharmacia Biotech) were equilibrated by washing with 0.5 ml of 50% TBS at 4^{o}C (10 minutes incubation, 5 minutes centrifugation at 2500 rpm 4^{o}C to remove supernatant) four times. The supernatant from the cell lysate was then loaded onto the equilibrated beads and rotated at 4^{o}C for 30 minutes. Supernatant was then removed at the end of binding step and was preserved for checking. The beads were then washed with 0.5ml of TBS-PMSF four times as described before. Finally the beads were suspended in 50µl of TBS-PMSF and 8 µl of glycerol was added for storage at -20^{o}C. 10 µl of beads with slurry was mixed with appropriate volume of 2x SDS-PAGE loading dye. Samples were boiled at 100^{o}C for 5 minutes, centrifuged at full speed for 5 minutes and analysed on SDS-PAGE.

4.14.4. Protein Purification by Immobilised Metal Ion Affinity Chromatography

Proteins expressed by using the QIAexpress (Qiagen) pQE expression vector system carry a stretch of six consecutive histidine residues (His-tag) fused to their amino terminal region allowing affinity purification using nickel ions immobilised on a metal chelation resin. Elution of the bound protein is performed by imidazole or by lowering the pH. Purification could be carried out under native conditions or, if the protein is insoluble, under denaturing conditions. The principals of the purification system and the basic protocols used are described in the QIAgen QIAexpress Handbook (1992).

A single colony of M15 strain of *E.Coli* transformed with pQE-C1D construct was inoculated in 10 ml of LB (supplemented by 50 µl/mg ampicillin and 25 µl/mg kanamaycin) and grown overnight at 37^{o}C. The starter culture was diluted to an O.D$_{600}$ around 0.2 in 100 ml of LB (with the appropriate antibiotics) and was grown at 37^{o}C until its O.D$_{600}$ reached to 0.75. IPTG was added to a final concentration of 0.5 mM and cells were grown further for 4 hours at 37^{o}C. The cells were harvested by centrifugation at 4000 rpm for 20 minutes at 4^{o}C; the pellet was then resuspended in Buffer B (8M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH adjusted to 8.0 using 10 M NaOH) at 5 ml Buffer B/gram weight of pellet. The cells were then stirred in a sterile beaker for one hour at room temperature. Meanwhile, 1.6.ml of a 50% slurry Ni-NTA resin (Qiagen) was loaded to a column with a flow rate of 10-15 ml/hr and was equilibrated by washing 10 column volumes of buffer B. The lysate was centrifuged at 10,000g for 15 minutes at 4^{o}C, and the supernatant was collected and was loaded onto the column pre-equilibrated in Buffer B. The column was then washed with Buffer B until the OD$_{280}$ of the flow-through was lower

than 0.01, and with Buffer C (8M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH adjusted to 6.3 using 37% HCl) until OD_{280} of the flow-through was lower than 0.01.

4.14.5. Protein Refolding

Each denatured proteins needs to be refolded according to a specially optimised protocol. Refolding is generally carried out by gradual dilution of the denaturing agents, together with careful reformation of the disulfide bridges.

Many proteins that are insoluble can be successfully refolded while immobilised on the Ni-NTA column. It may be that immobilising one end of the protein during renaturation prevents the formation of misfolded aggregates. The column containing the His-C1D was applied to a linear 6 M- 0 M urea gradient in 500 mM NaCl, 20% Glycerol, 20 mM Tris-Cl pH 7.4 over a period of 12 hours. The refolded C1D was kept immobilized on the Ni-NTA beads in 0.1x TE (containing protease inhibitors) for the Ni-NTA pull-down assays. Alternatively to use in the kinase assays, the folded C1D was eluted with the addition of 250 mM imidazole in 0.1X TE (containing protease inhibitors) and collected fractions were analysed by SDS PAGE. Fractions containing the recombinant protein were further analysed by western blotting to confirm the protein purification. Eluted proteins were kept in the same buffer at 4^{o}C till usage.

4.14.6. *In vitro* Transcription and Translation

The *in vitro* transcription and translation of target proteins were carried out using the TNT T7 Coupled Reticulocyte Lysate System (Promega), according to the manufacturer's instructions using [^{35}S]-methionine. Samples (5µl) of the [^{35}S]-methionine-

labelled translation products were electrophoretically separated on a SDS-Polyacrylamide gel and the bands were visualised by autoradiography.

4.14.7. Pull-Down Assays

4.14.7.1. Ni-NTA Pull-Down Assay

Before the binding reaction, Ni-NTA bound to the protein of interest was equilibrated by washing (10 minutes incubation followed by centrifugation at 2500 rpm for 5 minutes and disposal of the supernatant) three times with binding buffer (20% glycerol, 100 mM NaCl, 0.1x TE pH 8.0, 0.1% NP-40, 1x Complete protease inhibitors). 20 µl of radiolabelled translation product and Ni-NTA bound to the protein of interest were incubated in 200 µl of Binding buffer containing 1mM DTT and 30 µg BSA for one hour at room temperature on a rotating wheel. At the end of the incubation the beads were collected by centrifugation at 2500 rpm for 5 minutes and the supernatant was removed. Then the beads were washed four times with 1 ml of wash buffer (20% glycerol, 100 mM NaCl, 0.1xTE pH 8.0, 0.3% NP40, 1x complete protease inhibitors) at 4^{o}C as described before. After the last wash the supernatant was discarded and the beads were resuspended in 20 µl of 2x SDS-PAGE loading buffer, boiled, centrifuged at 13000 rpm for 5 minutes and loaded on 10% SDS-PAGE. After electrophoresis, the gel was soaked in fixing solution (50% methanol, 10% glacial acetic acid, 40% ddH_2O) for 30 minutes. After pouring off the fixing solution, for increased detection sensitivity the gel was soaked in Amplify Reagent (Amersham) for 30 minutes and finally soaked in 7% acetic acid, 7% methanol, 1% glycerol for 5 minutes to prevent the gel from cracking during drying. The gel was placed on a sheet of Whatman 3MM filter paper, covered with plastic wrap and

dried at 65°C for 2 hours under a vacuum using a conventional gel drier (Savant). After drying, the gel was exposed to the film.

Alternatively Ni-NTA pull-downs were performed in the following binding buffers: Buffer A (100 mM NaCl, 20% glycerol, 20 mM Tris-Cl pH 7.5, 0.3% NP40, 1x complete protease inhibitors), Buffer B (50 mM NaPhosphate pH 8.0, 100 mM NaCl, 12.5 mM $MgCl_2$, 0.3% NP40, 1x complete protease inhibitors) or Buffer C (25 mM HEPES pH 7.6, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 20% glycerol, 0.3% NP40, 100 mM KCl, 1x complete protease inhibitors).

4.14.7.2. GST Pull-Down Assay Using Immobilized GST-C1D

Before the binding reaction, glutathione sepharose-beads bound to GST and GST-C1D were equilibrated by washing three times with Z buffer (25 mM HEPES pH 7.5, 12.5 mM $MgCl_2$, 20% Glycerol, 0.1% NP-40, 150 mM KCl, 1x complete protease inhibitors) as described before. 20 µl of glutathione sepharose beads bound to the protein of interest was preincubated in 200 µl of Z buffer containing 1 mM DTT and 30 µg BSA with gentle rocking for 10 minutes at room temperature. Then, 20 µl of radiolabelled translation product was added onto the beads, and incubated at room temperature for 1 hour with gentle rocking. At the end of incubation, samples were centrifuged at 2500 rpm for 5 minutes and the supernatant was removed. The beads were then washed four times with 1 ml of NETN (20 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP40, 1x complete protease inhibitor) at 4°C. Samples were then analysed on SDS-PAGE and the gel was dried and exposed to film as described in the previous section.

Alternatively GST pull-down assay was performed through another route. Instead of using radiolabelled translation product, lysate of COS7 cells transiently transfected with the plasmid expressing the protein of interest was incubated with the beads. Cells were collected as described in immunoprecipitation section and were resuspended in 50 µl of Lysis Buffer (25 mM HEPES pH 7.5, 12.5 mM $MgCl_2$, 20% Glycerol, 0.5% NP-40, 150 mM KCl, 1x complete protease inhibitors) and were incubated on a rotating wheel for 30 minutes. Following incubation cell lysates were spun down at 10000g for 10 minutes to remove the insoluble cell debris. The supernatant, which is the total cell lysate, was removed into a new tube and diluted to 250µl with the dilution buffer (25 mM HEPES pH 7.5, 12.5 mM $MgCl_2$, 20% Glycerol, 150 mM KCl, 1x complete protease inhibitors). The cell lysate was then added onto the glutathione sepharose beads bound to the protein of interest and was incubated for 1 hour at room temperature on a rotating wheel. Beads were washed as before and were subjected to electrophoresis. After electrophoresis, proteins were transferred to a nitrocellulose membrane and the immunological detection of the immobilized proteins was performed.

4.14.8. Kinase Assays

Two nanograms of purified DNA-PKcs together with 5 ng of Ku was incubated with 100 ng of purified His-C1D and 100 ng of linear DNA in the presence of 20 µl of Z' buffer (25 mM HEPES pH 7.9, 50 mM KCl, 10 mM $MgCl_2$, 20% glycerol, 0.1% NP40, 1mM DTT, and 200 µM ATP) for 10 minutes on ice and the reaction was started by adding 10 µCi of [γ^{32}-ATP]. Following incubation at 30^{o} C for 30 minutes, phosphorylated proteins were subjected to 15% SDS-PAGE and visualised by autoradiography.

When immuno-complex protein kinase assays were performed, immunoprecipitates were prepared as described before and were washed three times with Z' buffer without ATP. After the last wash, 10 µl of immunoprecipitates was added to the kinase reaction mixture and kinase assay was performed as described before.

4.15. RADIOCHEMICALS

[^{35}S]methionine and [γ-^{32}P]ATP were supplied by Amersham International and DuPont NEN.

4.16. RESTRICTION AND DNA MODIFYING ENZYMES

All enzymes were obtained from GibcoBRL, except *Taq* DNA polymerase and related components from Sigma, and used according to the instructions of the manufacturers.

4.17. CHEMICALS, KITS AND SPECIAL CONSUMABLES

All chemicals were purchased from Sigma, Difco, Carlo-Erba, or Merck and plastic disposables from Costar or Greiner Labortechnik. Plasmid purification kit was supplied by Macherey-Nagel, Ni-NTA resin for histidine tagged protein purification by Qiagen, and TNT coupled reticulocyte lysate kit by Promega. FuGene6 transfection reagent and complete protease inhibitor cocktail were obtained from Roche Molecular Biochemicals. IPTG was obtained from MBI Fermentas, ONPG from Sigma, ECL from Amersham Life Science, X-ray films from Kodak. Anti-HA and Anti-TAFI 48 antibodies were supplied by SantaCruz and all secondary HRP conjugated antibodies were supplied by Sigma.

4.18. EQUIPMENT

The following equiments were routinely used during this study: Automatic pipettes (Rainin), filter and 3MM paper (Whatman), GeneAmp PCR System 9600 (Perkin Elmer), heating blocks (Stuarts Scientific), benchtop centrifuge (Heraus Instruments), Avanti J-25I centrifuge (Beckman), gel tanks for agarose (E-C Apparatus Corporation), Mini-PROTEAN 3 Cell (BioRad), spectrophotometer DU640 (Beckman), power supply PAC 300(BioRad), pH meter (Beckman), UV transilluminator (Herolab), Slab Gel Drier (Savant), Mini Trans-Blot (Biorad).

RESULTS

5. RESULTS

5.1. INTRODUCTION

Identification of the biological functions of the nuclear matrix protein C1D is important, as it will possibly provide evidence of how nuclear matrix is involved in regulation of cellular processes. An equally important issue is to demonstrate the role of DNA-PK in these events in connection with the nuclear matrix. In an attempt to identify the biological functions of C1D, a yeast two-hybrid system was employed to screen a human B-lymphocyte cDNA library using the full-length open reading frame of C1D as bait (Dincer et al. Bilkent University, unpublished). In this system, a protein of interest is expressed in fusion with a DNA-binding domain of a transcription factor lacking a transcription activation domain (bait). The yeast strains used for this purpose contain reporter genes (LacZ and/or HIS3) with binding sites for this DNA-binding domain. When expressed in the yeast, the bait plasmid will be able to bind to these specific sequences in the promoter region of the reporter gene through its DNA-binding domain. However, expression of the LacZ or HIS3 will not occur as this protein-DNA complex lacks an activation domain. The yeast strain carrying the bait plasmid is then transformed with a cDNA library (or another protein of interest) expressed in fusion with an activation domain. Thus, if any of the proteins encoded by the cDNA interacts with the bait protein, transcriptional activation will occur and the expression of the reporter genes is measured by means of the β-galactosidase activity produced or growth capacity of yeast cells in the absence of HIS3, both of which are direct indicators of interaction of two proteins.

The yeast two-hybrid screening using C1D as bait, revealed five cDNA groups mainly encoding proteins involved in recombination and repair. A search of the GenBank database revealed that one group of overlapping sequences that derived from the same cDNA encoded the TAFI 48 subunit of SL1. In this study, we aimed to verify this interaction and confirm that not only the partial cDNA sequences but the full-length TAFI48 can also interact with C1D in various different *in vivo* and *in vitro* systems.

5.2. CLONING OF TAFI 48 IN VARIOUS EXPRESSION PLASMIDS

The TAFI 48 full-length open reading frame was amplified using PCR and was cloned into yeast, bacterial, mammalian and baculovirus expression plasmids as described in Materials and Methods (4.8.7.). The recombinant plasmids were checked by appropriate restriction enzymes. Table 5.1 depicts the expression plasmids and the expected sizes using the corresponding enzymes. A sample digest is presented in fig.5.1.

Table 5.1: Restriction Mapping of the Constructs

VECTOR	DIGEST	EXPECTED FRAGMENTS (bp)
TAFI 48-pACT2	*Eco*RI	760, 192
TAFI 48-pQE30	*Hind*III	610, 171
TAFI 48-T7-myc-pLink	*Eco*RI	192
TAFI 48-pCMV	*Eco*RI	400, 192
TAFI 48-pcDNA3.1	*Hind*III	622, 610
TAFI 48-pBlueBacHis2	*Hind*III	610

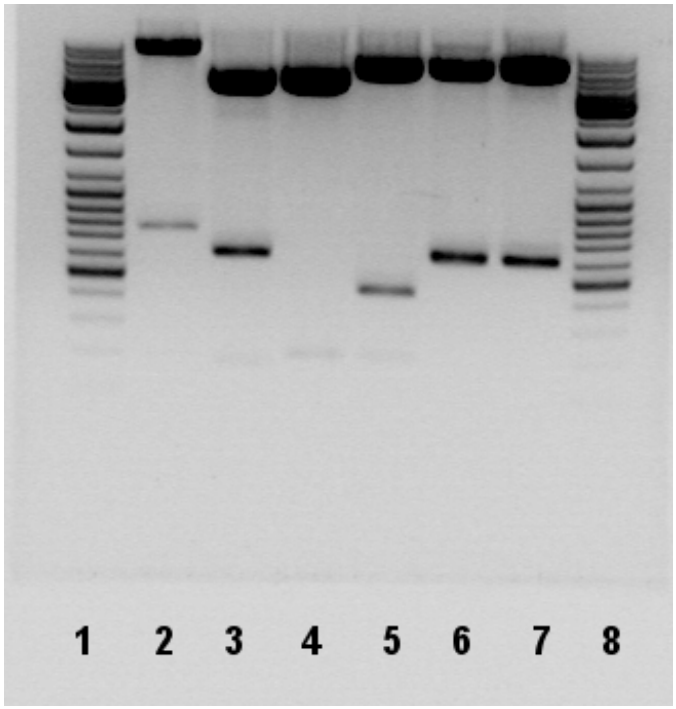

Figure 5.1: Sample digest result

Lanes:

1- GeneRuler DNA Ladder Mix
2- TAFI 48-pACT2 digested with *Eco*RI
3- TAFI 48-pQE30 digested with *Hind*III
4- TAFI 48-T7-myc-pLink digested with *Eco*RI
5- TAFI 48-pCMV digested with *Eco*RI
6- TAFI 48-pcDNA3.1 digested with *Hind*III
7- TAFI 48-pBlueBacHis2 digested with *Hind*III
8- GeneRuler DNA Ladder Mix

5.3. CONFIRMATION OF C1D/TAFI 48 INTERACTION IN YEAST USING ONPG ASSAY

It is always possible that the partial cDNA clones obtained as a consequence of a yeast two-hybrid screening could belong to another protein and/or might be translated to an unrelated polypeptide. It is essential therefore, to confirm the interaction using the full-length proteins. To establish this, the full-length open reading frame of TAFI 48 was expressed in yeast as an activation domain tagged fusion protein (this fusion is referred to as AD-TAF 48). AD-TAF 48 (in pACT2) was transformed into the strain L40 together with baits (in pBTM116) comprising the LexA DNA-binding domain (DBD) alone, DBD-Lamin (DBD-La), DBD-Daughterless (DBD-Da), or DBD-C1D and the level of β-galactosidase activity from the integrated LacZ reporter was determined in each case using the ONPG assay. As seen in Fig.5.2, TAFI 48 interacted specifically with C1D since no interaction was detected with DBD alone, or with heterologous fusion proteins, DBD-Lamin, or DBD-Daughterless.

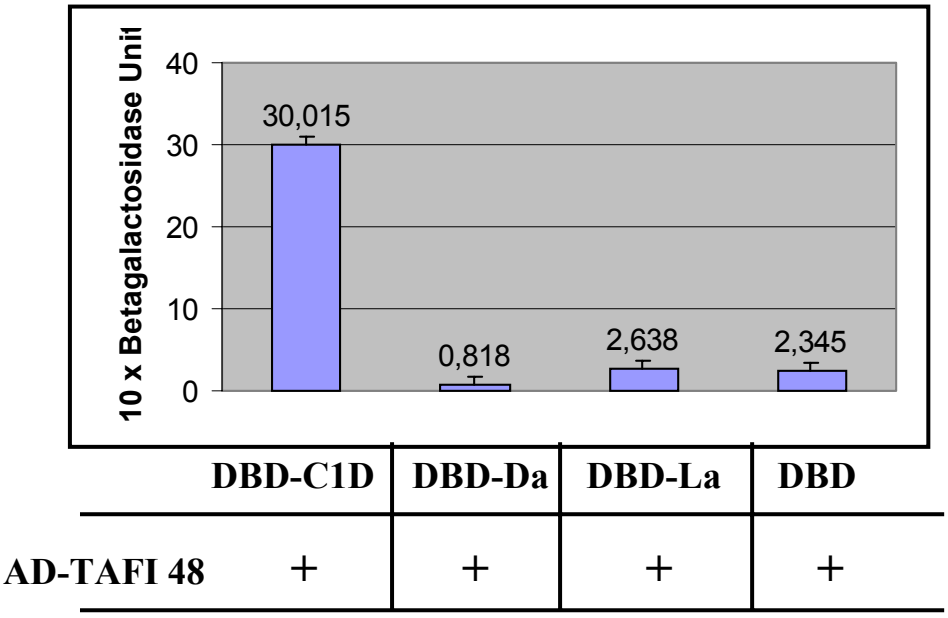

Figure 5.2: Yeast two hybrid assay. β-galactosidase activity was measured using ONPG as a substrate to determine the specificity of the interaction between C1D and TAFI 48.

5.4. *IN VITRO* INTERACTION ASSAYS

Having confirmed that C1D and TAFI 48 interact specifically in yeast, *in vitro* assays were performed to determine whether these two proteins would also interact *in vitro*. For this purpose, one approach would be to express and purify TAFI 48 in bacteria and check its interaction with the purified C1D. However, despite using various conditions including different IPTG concentrations, induction at different temperatures for varying time courses and by using different bacterial strains, it was not possible to express TAFI 48 in bacteria. Therefore, another approach was taken towards immobilizing C1D on a column and then performing the pull-downs with the *in vitro* transcribed and translated TAFI 48 product. Previous studies demonstrated that the bacterially expressed C1D was

insoluble, however, could be purified under denaturing conditions followed by refolding in the presence of DNA (Yavuzer et al. 1999). In other words, it was impossible to keep C1D in solution unless it was refolded on DNA. In these old purification schemes, the denatured C1D was first eluted from the column and then was refolded slowly against a urea gradient in the presence of linear or supercoiled DNA. However, for our purposes, C1D had to be refolded and kept in solution while it was still immobilized on Ni-NTA column. It was also important to obtain purified C1D without having the DNA around as this could give rise to non-specific interactions or interaction of two proteins through DNA rather than direct protein-protein interaction.

5.4.1. Purification of His-Tagged C1D Protein Under Denaturing Conditions

C1D-pQE30 (provided by U. Yavuzer) was expressed in bacteria and purified under denaturing conditions as described in materials and methods (Section 4.14.4.). Unlike the methods used in previous purification methods, the denatured protein was kept bound to the column and was subjected to a linear urea gradient without adding DNA into the refolding buffer. The refolded C1D that was immobilized on Ni-NTA column was then stored in 0.1x TE (containing protease inhibitors) for pull-down assays. A little amount of immobilized C1D was subjected to SDS-PAGE to check the purity and amount of the bound protein. As seen in Fig.5.3, a single protein band of expected molecular size (18 kDa) to C1D was detected, suggesting that the purification had been successful and that enough amount of a soluble protein has been obtained.

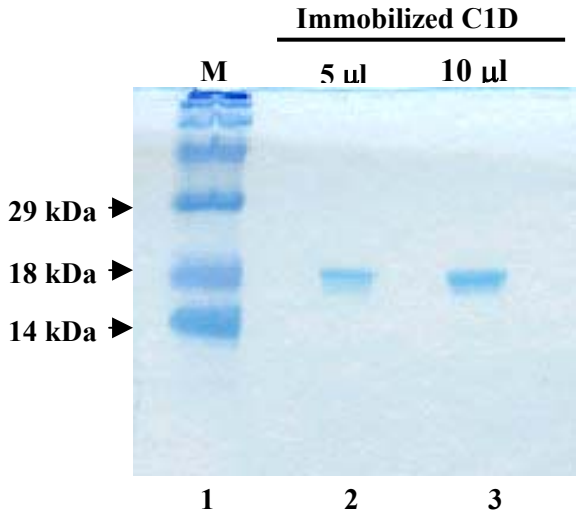

Figure 5.3: Ni-NTA immobilized C1D. C1D immobilized on Ni-NTA column using the modified method was detected by Coomassie staining after resolving the protein complexes on SDS-PAGE. M, is the protein size marker.

Next step was to demonstrate whether this protein would still be soluble after being eluted from the column. Another equally important issue was to show that this was the actual C1D protein and not any other irrelevant protein with a similar size to C1D. For this purpose, the refolded protein was eluted from the column and the protein fractions were resolved by SDS-PAGE followed by western blotting using a polyclonal antibody raised against C1D. Fig. 5.4 demonstrates that anti-C1D antibody recognizes the protein fractions eluted from the column and that the new purification scheme that was used to refold C1D in the absence of DNA has been successful. A protein band around 32 kDa indicates that eluted fractions contain C1D as concatamers that are extremely stable dimers of C1D.

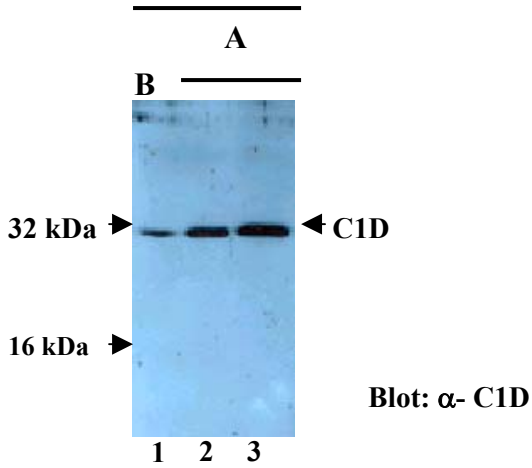

Figure 5.4: Western blot detection of C1D. C1D bound to the Ni-NTA column was eluted by Imidazole and the protein fractions were subjected to western blotting using an antibody raised against C1D. Lane 1 is 30 µl of protein from fraction B, lanes 2 and 3 contain protein from fraction A of 15 µl and 30 µl, respectively.

5.4.2. Ni- NTA Pull-Down Assay

C1D that has been purified and refolded under the conditions described above was then used for *in vitro* interaction assays. For this purpose TAFI 48 was *in vitro* transcribed and translated using the TnT Reticulocyte lysate system in a total volume of 50 µl. Because S^{35}-methionine is used in this process, it is possible to detect the proteins by directly exposing the gels to autoradiography.

Ni-NTA beads alone (Fig. 5.5 lane 4) or beads containing the immobilized C1D (lane 3) were incubated with the *in vitro* transcribed and translated TAFI 48 product and pull-down assays were performed as described in Materials and Methods (section 4.14.7.1). Although there was a significant binding of TAFI 48 to C1D (lane 3), similar amount of

TAFI 48 was also seen to be bound by the Ni-NTA beads alone (lane 4), suggesting that the *in vitro* interaction detected under these assay conditions is not specific. Despite several attempts by using more stringent conditions, it was not possible to eliminate the affinity of TAFI 48 to Ni-NTA beads. Although TAFI 48 does not contain a stretch of histidine amino acids as such, it is possible that a small amino acid region within the TAFI 48 could recognize Ni-NTA and bound non-specifically. Alternatively, the reticulocyte-lysate could be the reason of this non-specificity.

Therefore, it was decided to use another column to immobilize C1D, with the hope that TAFI 48 would not bind non-specifically.

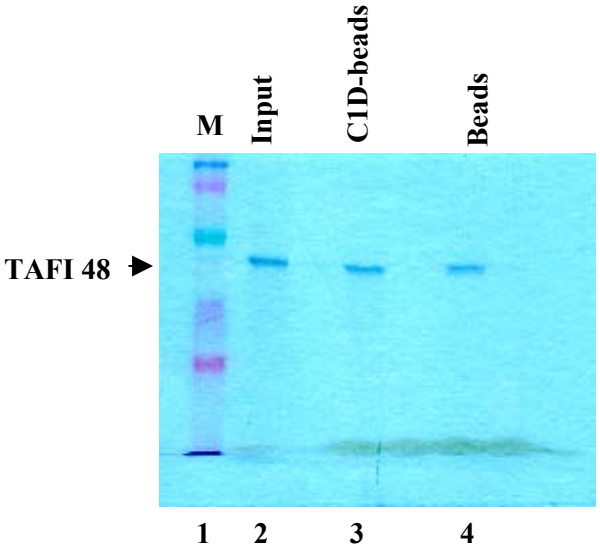

Figure 5.5: Ni-NTA pull-down assay. *In vitro* transcribed and translated TAFI 48 (lane 2) was incubated with C1D immobilized on Ni-NTA (C1D-beads) (lane 3) and Ni-NTA beads alone (lane 4) and pull-down assay was performed. The bands were visualized by autoradiography. M, is the protein size marker.

5.4.3. Purification of GST-C1D Protein Under Non-denaturing Conditions

For this new approach, widely used glutathione sepharose system for *in vitro* interaction assays was chosen. As a first attempt, C1D was expressed in bacteria in fusion with GST (GST-C1D, provided by U. Yavuzer). GST on its own was also expressed and purified under the same conditions as GST-C1D. Interestingly, C1D expressed in fusion to GST is soluble (Yavuzer et al. 1998). Figure 5.6 shows the purification result where the proteins bound to sepharose beads were mainly GST and GST-C1D, with expected sizes of 27.5 kD and 45.5 kD respectively.

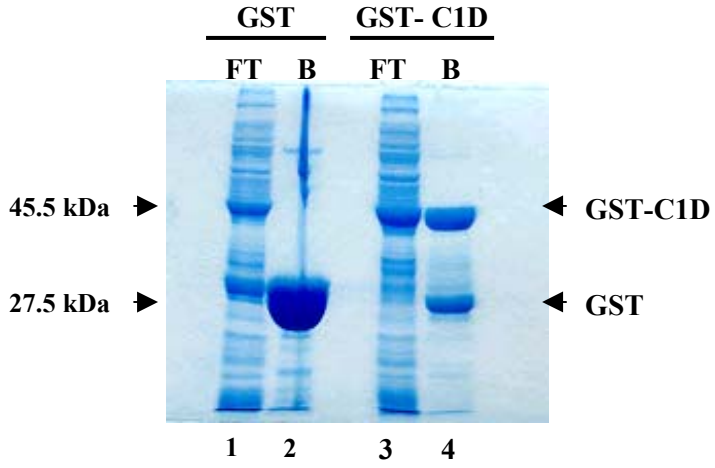

Figure 5.6: Purification of GST and GST-C1D fusion proteins. GST- C1D and GST on its own were expressed in bacteria and bound to glutathione sepharose beads. The flow-through (FT) from each column was spared after the first wash for detection of unbound proteins. The bound proteins (B) and FT fractions were resolved on SDS-PAGE and visualized by Coomassie staining.

5.4.4. GST Pull-Down Assays Using IVTT Product of TAFI 48.

The purified GST and GST-C1D were immobilized on glutathione beads and were incubated with the *in vitro* transcribed and translated TAFI 48. After extensive washing, the protein complexes were resolved on SDS-PAGE and analyzed. It was once again seen that TAFI 48 bound to both GST and GST-C1D, albeit binding being about 5-fold stronger to C1D than to that GST alone (Fig. 5.7, compare lanes 2 and 3). Although this experiment suggested that these two proteins interact *in vitro* as well, clearly the IVTT product of TAFI 48 somehow shows affinity towards both Ni-NTA and less strongly to GST. Since this could be an artifact due to reticulocyte lysate, it was decided to obtain TAFI 48 via another route rather than using *in vitro* transcription and translation system.

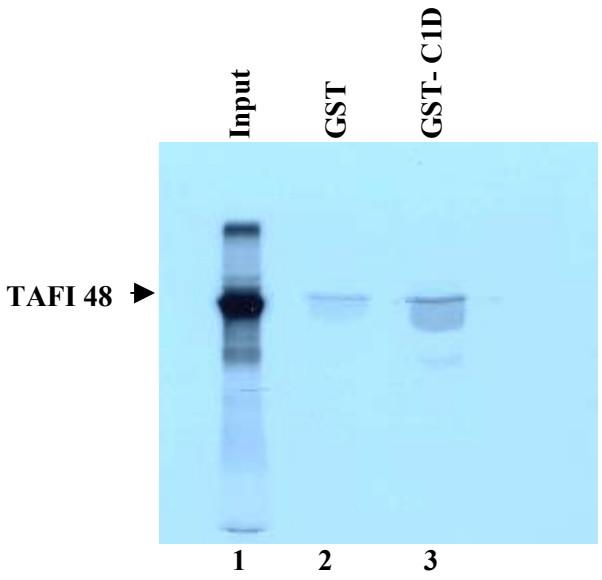

Figure 5.7: GST pull-down assay using IVTT product of TAFI 48. IVTT product of TAFI 48 was incubated with GST (lane 2) and GST-C1D (lane 3) immobilized on glutathione beads and the complexes were resolved on SDS-PAGE and visualized by autoradiography.

5.4.5. GST-Pull-Down Assay Using TAFI 48 Expressed in Mammalian Cells

For this purpose, a mammalian cell line, COS7, was transiently transfected with a plasmid expressing TAFI 48. As an HA-epitope was placed to the N-terminal region of TAFI 48, it was possible to detect the expression of the protein using an antibody raised against the HA epitope. After 30 hours following transfection, total cell lysates were prepared from cells transfected with TAFI 48-pCMV. These lysates were then incubated with the immobilized GST and GST-C1D and pull-down assays were performed as described before. After resolving the protein complexes on SDS-PAGE, the proteins were transferred to nitrocellulose and western blotting was performed using an antibody against HA epitope. As seen in Fig. 5.8 lane 1, the immobilized GST alone did not bind any protein from the cell lysate. However a protein band of expected molecular size for HA-TAFI 48 was readily detectable when GST-C1D was incubated with the TAFI 48-pCMV transfected cell lysate (lane 2). To confirm that observed protein band is HA-TAFI 48, the lysates from untransfected and TAFI 48-pCMV transfected cells were immunoprecipitated using anti-HA antibody. The protein complexes obtained by immunoprecipitation were also run along the GST/GST-C1D pull-downs and probed with anti-HA antibody. As presented in lane 3, the protein bound to GST-C1D in lane 2, is not present in lysates from untransfected cells but is detectable in the immunocomplexes precipitated from the transfected cell lysate, indicating that the protein complex that was bound to GST-C1D is HA-TAFI 48.

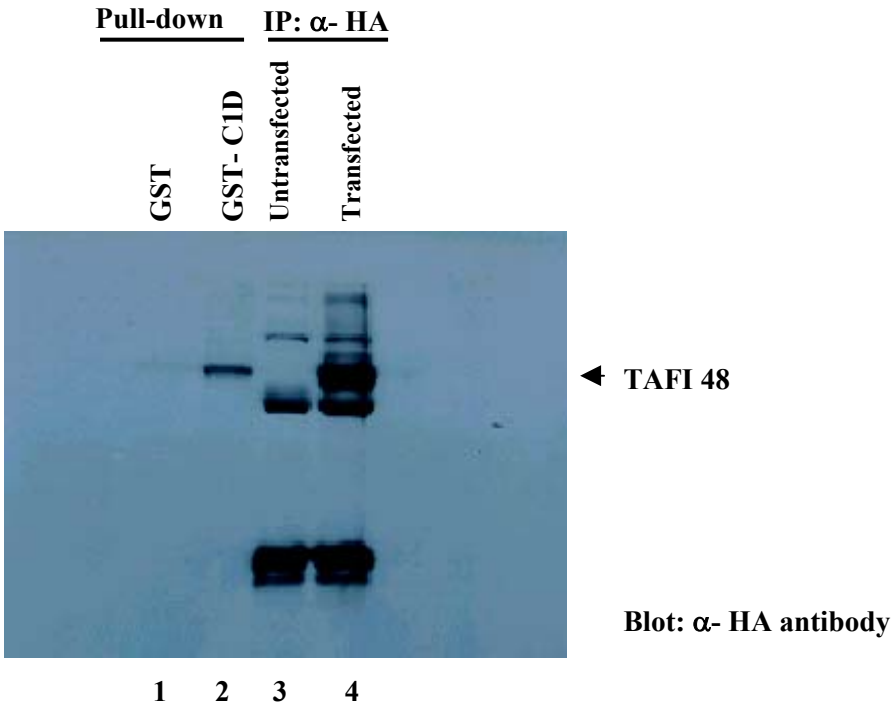

Figure 5.8: GST pull-down assay using TAFI 48 expressed in mammalian cells. Lanes 1 and 2 are the immobilized GST and GST-C1D on glutathione beads incubated with the cell lysate from HA-TAFI 48 transfected cells. Lanes 3 and 4 are the cell lysates from untransfected and HA-TAFI 48 transfected cells immunoprecipitated with anti-HA antibody. The complexes were resolved on SDS-PAGE, transferred to nitrocellulose and probed with anti-HA antibody.

5.5. KINASE ASSAYS

The *in vivo* and *in vitro* interaction between one of the subunits of SL1, which is involved in Pol I transcription, and C1D is potentially interesting because of their mutual connection with DNA-PK. Since the molecular mechanisms underlying the role of DNA-PK in the repair of DSBs are not known, it is possible that this new interaction might provide some evidence towards identification of these mechanisms. It was important therefore, to set up a functional assay to have an idea about the possible biological

consequences of interaction between C1D and TAFI 48. One possible mechanism, DNA-PK could phosphorylate TAFI 48 either directly or by the aid of C1D. However, before performing this experiment it was necessary to demonstrate that C1D protein that has been purified using the modified method behaves similar to C1D obtained by traditional methods. Since C1D is known to be a very efficient substrate for DNA-PK, *in vitro* DNA-PK kinase assays were performed using C1D purified and refolded in the absence of DNA. In this assay purified DNA-PKcs, Ku (kindly provided by S.Jackson) and C1D proteins were incubated in the presence of [γ^{32}-ATP]. As being a kinase, DNA-PKcs together with Ku would transfer the radioactively labeled ATP onto C1D, enabling us to detect the protein by autoradiography, if it is being phosphorylated.

In agreement with the previous studies, DNA-PKcs did not have any kinase activity in the absence of Ku subunit or DNA (Fig. 5.9 lanes 1 and 2) and when linear DNA was included, the autophosphorylation was detected (lane 3). On the other hand, the C1D that was purified in the absence of DNA was phosphorylated as efficiently as the C1D purified by other methods using DNA for refolding.

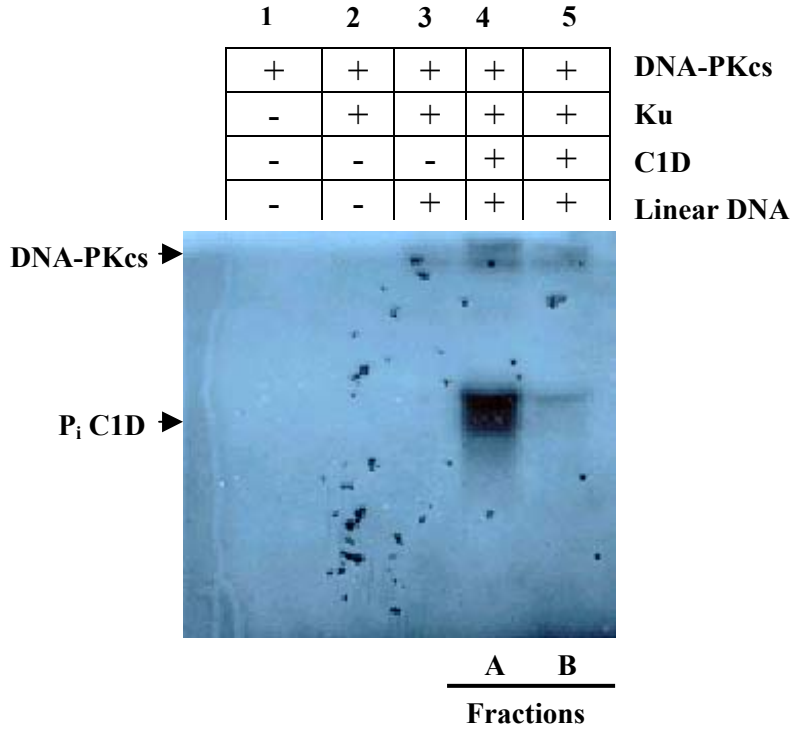

Figure 5.9: *In Vitro* kinase assay. C1D purified with the modified method was incubated with DNA-PK in the presence of [γ^{32}-ATP] and the phosphorylated proteins were detected by autoradiography. The autophosphorylated DNA-PKcs and phosphorylated C1D is indicated by arrow heads.

After having demonstrated that C1D purified with our modified method is also a substrate for DNA-PK, TAFI 48 was checked for being phosphorylated by DNA-PK in the absence or presence of C1D. For this purpose, the COS7 cells were transiently transfected with TAFI 48 and cell lysates were immunoprecipitated using anti-HA antibody as described before. The immune-complex was then subjected to kinase assay in the presence of [γ^{32}-ATP]. As a positive control, the well-known substrate for DNA-PK was also included (Fig 5.10 lane 6). The cell lysate from cells expressing the HA-epitope was immunoprecipitated with anti-HA antibody and was also subjected to kinase assay to

demonstrate that the reaction is not inhibited by the cell lysates. As demonstrated in Fig 5.10, both the autophosphorylation of DNA-PKcs (lanes 1 and 2) and phosphorylation of C1D (lane 3) could be detected and the presence of cell lysate immunoprecipitated with the HA-antibody did not affect the phosphorylation of either of the proteins (lanes 2 and 3). In lane 4, anti-HA antibody immunoprecipitate from HA-TAFI 48 transfected cell lysates were assayed for phosphorylation by DNA-PK. As clearly seen, DNA-PK was not able to phosphorylate TAFI 48 on its own. Next, the same reaction was repeated in the presence of C1D, and again DNA-PK failed to phosphorylate TAFI 48, although phosphorylation of C1D was readily detectable (Lane 5). Therefore, it was concluded that, C1D purified by our modified method was an efficient substrate for DNA-PK, however, DNA-PK failed to phosphorylate TAFI 48 either in the absence or presence of C1D.

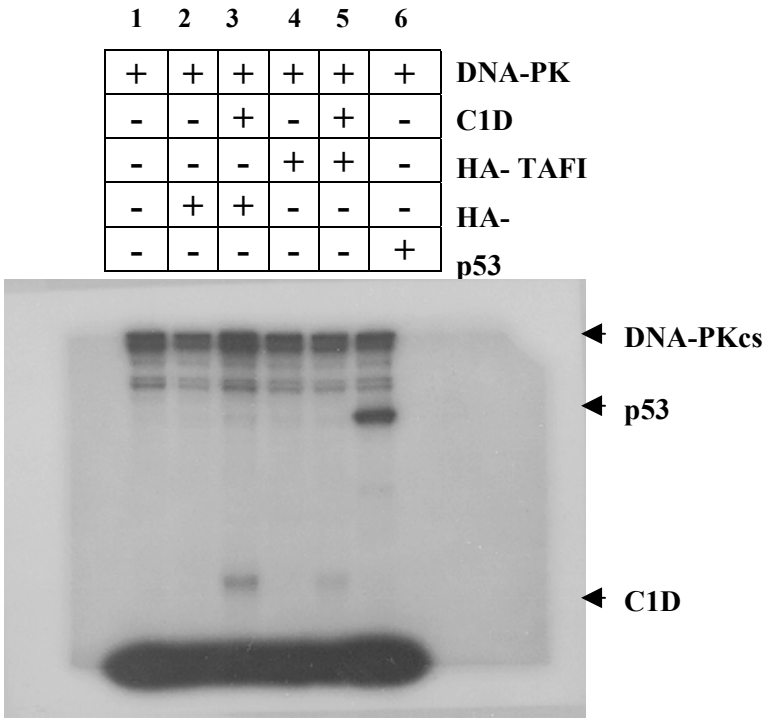

Figure 5.10: Immune-complex Kinase Assay. COS 7 cells were transiently transfected with plasmids expressing either HA-TAFI 48 or HA-epitope. The cell lysates were immunoprecipitated with anti-HA antibody and the immune-complexes were subjected to kinase assay using purified DNA-PK and C1D. All reactions contained 50 ng of linear plasmid DNA.

5.6. TAFI 48/C1D INTERACTION IN MAMMALIAN CELLS

We also wished to test whether C1D and TAFI 48 are capable of interacting with one another in mammalian cells. COS7 cells were transiently transfected with a plasmid expressing HA-epitope tagged C1D (C1D-pCMV, provided by U. Yavuzer).. 30 hours following transfection, cell lysates were prepared from transfected and untransfected cells and immunoprecipitated with an anti-HA antibody. The immunoprecipitates were then analyzed by western blotting using anti-TAFI 48 antibody. As shown in Fig. 5.11A, TAFI

48 immunoprecipitated with HA-C1D, although the interaction seemed to be rather weak. To demonstrate that, this weak interaction is not due to the inefficient immunoprecipitation of HA-C1D, the membrane was stripped and re-probed with the anti-HA antibody. Fig.5.11B demonstrates that HA-C1D is immunoprecipitated very efficiently from the transfected but not untransfected cells. Another reason for this weak interaction could be that the band detected with the anti-TAFI 48 antibody is not specific and the antibody could be recognizing an irrelevant protein from the cell lysate. To eliminate this possibility, the membrane was stripped for the third time and re-probed with anti-TAFI 48 antibody that has been incubated with an antibody specific peptide for 2 hours. If the detected protein band in Fig.5.11A was really TAFI 48, then we would expect this band to disappear after probing with the neutralized antibody. In Fig 5.11C, it is shown that, although there was a decrease in the intensity of the band, it did not disappear completely. Nevertheless, because the neutralized antibody inhibited the detection of TAFI 48 to an extent, it was concluded that C1D and TAFI 48 may interact in mammalian cells, however, rather weakly under physiological conditions.

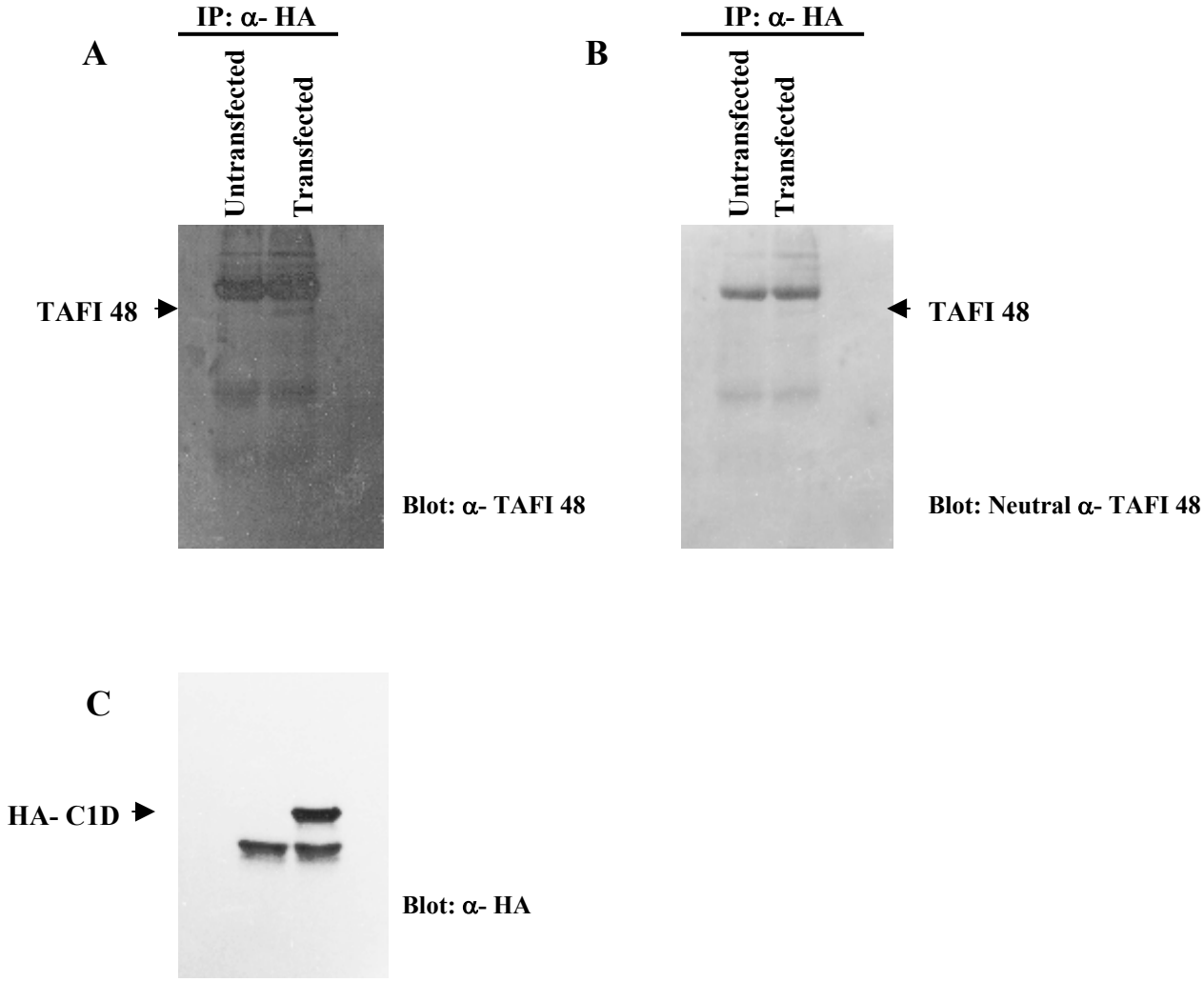

Figure 5.11: Immunoprecipitations from mammalian cells. **A)** Untransfected and HA-C1D transfected cells were immunoprecipitated with the indicated antibody and probed with the anti-TAFI 48 antibody to check for the interaction between C1D and the endogeneous TAFI 48. **B)** The mebrane was stripped and re-probed with anti-HA antibody. **C)** The mebrane was re-stripped and probed with the neutralized TAFI 48 antibody.

DISCUSSION

6. DISCUSSION

First described by Brown in 1831, the cell nucleus is one of the best known but least understood of cellular organelles. Current evidence suggests that the nucleus is spatially ordered by attachments to a non-chromatin nuclear structure, the nuclear matrix (Nickerson et al. 1995). The nuclear scaffolding juxtaposes factors, enzymes, and other macromolecules in appropriate spatial domains of the nucleus, thereby effecting an efficient assembly of the complexes involved in replication, transcription, and RNA processing. The nuclear matrix binds most nuclear RNA and organizes chromatin attachment sites responsible for chromatin loops. Many of the intermediates of nucleic acid metabolism, together with regulatory and catalytic factors, remain complexed to the nuclear matrix when separated from chromatin. This and other evidence suggest that nucleic acid synthesis and processing are both nuclear matrix-bound processes (Nickerson et al. 1995; Van Driel et al. 1995; Berezney et al. 1995).

Nuclear matrix contains 10-25% of the total nuclear protein and nuclear matrix proteins are the nonhistone proteins that are tightly bound to DNA even after treatment with harsh denatures (Neuer et al. 1983). These nonhistone proteins have a heterogeneous profile and include cell type and differentiation state-specific proteins as well as common proteins termed the nuclear matrins.

The nuclear matrix protein C1D is the first of such non-histone proteins to have been characterized at the sequence level (Nehls et al. 1998). In an independent research, C1D was found to be associated with the transcriptional repressor RevErb and the nuclear co-receptors, indicating that it could act as a component of the complex involved in

transcriptional repression (Zamir et al. 1997). C1D has also been shown to induce apoptosis in a p53 dependent manner (Rothbarth et al. 1999) in agreement with the function of DNA-PK as an upstream effector for p53 activation in response to IR, linking DNA damage to apoptosis (Wang et al. 2000). Together with these observations when its interaction with DNA-PK and possible involvement in DNA repair is considered, we can say that C1D is a multifunctional protein. Attempts towards identification of the biological function of C1D led to the isolation of TAFI 48 as an interacting protein with C1D. Because the Pol I transcription is repressed by DNA-PK by an unknown, but phosphorylation dependent mechanism, we believe that this interaction will provide a starting point towards revealing the molecular mechanisms DNA-PK undertakes while performing its functions.

In this study, we demonstrated that C1D and TAFI 48 interact in yeast, in mammalian cells and *in vitro* specifically, although the interaction between C1D and TAFI 48 seems to be weak with respect to other C1D interacting proteins.

TAFI 48 is a good candidate for recruiting modifying complexes to Pol I promoters because the binding of TAFI 48 to TBP is sufficient to block or conformationally alter the DNA binding surface of TBP in a manner that prevents recognition of the TATA-box element (Beckmann et al. 1995), which means that TAFI 48 is the major determinant in Pol I promoter selectivity. In addition, TAFI 48 serves as a target for interaction with UBF and is required to form a transcriptionally active SL1 complex responsive to UBF *in vitro* (Beckmann et al. 1995).

A weak interaction between C1D and TAFI 48 under normal physiological conditions would be meaningful if this interaction is expected to play a role in repression of Pol I transcription in response to certain exogenous stimuli such as γ-irradiation.

The data obtained so far enables us to propose two hypothetical models on how polymerase I transcription could be repressed by DNA-PK. In one model, the normally weak interaction between C1D and TAFI 48 would be enhanced in response to DNA damaging agents inducing DNA double strand breaks. Since DNA-PK is activated upon γ-irradiation and can phosphorylate C1D *in vitro*, it is possible that in response to DNA damage DNA-PK may get activated and as a consequence may phosphorylate C1D *in vivo*. This post- translational modification may enhance the affinity of C1D towards TAFI 48. In this work, chemicals such as MMS or etoposide were used to induce DNA double strand breaks, with the hope that the weak interaction detected in co-immunoprecipitation experiments could be enhanced. The problem with these experiments is that the dose and duration of the application of the chemical and also the time point when the DNA double strand breaks are induced vary according to the cell type. In our case although many different conditions were used, it was not possible to induce the interaction between C1D and TAFI 48 any further. Nevertheless, this does not rule out the possibility that C1D and TAFI 48 may interact in response to agents inducing DNA double strand breaks. Application of direct X-rays using a gamma irradiation source may overcome this problem. Unfortunately, this experiment was not performed as an irradiation source is not available in this department. The DNA-PK induced interaction between C1D and TAFI 48 in response to DNA damage may be important as this complex may hinder the formation of SL1 on the the rRNA promoter and thus, inhibit polymerase I transcription.

Alternatively, C1D/TAFI 48 interaction may act to recruit activated DNA-PK to rRNA synthesis sites and DNA-PK may then phosphorylate C1D, TAFI 48 or another subunit of SL1 to inhibit the transcription. TAFI 48 is a good candidate for phosphorylation by DNA-PK as it is possible that phosphorylation of TAFI 48 may disrupt its interaction with UBF so that phosphorylated SL1 no more binds to rRNA promoter. However, using the *in vitro* kinase assay with the highly purified DNA-PK, we were not able to show the phoshorylation of TAFI 48 by DNA-PK. Although the possibility still exists that this phosphorylation event may take place *in vivo*, it is equally possible that DNA-PK may target another subunit of SL1. It is already known that that the transcription of ribosomal RNA genes by RNA Polymerase I is inhibited by cdc2/cyclinB mediated phosphorylation of TAFI 110 (Heix et al., 1998). It would be of great interest to see if this subunit is also regulated by DNA-PK in response to DNA damaging agents and hence results in inhibition of Pol I transcription.

On the contrary to the first model where an indirect role for DNA-PK in repression of Pol I transcription has been suggested, in the second model, a more direct role has been proposed. Nevertheless, these two models may not necessarily be mutually exclusive. In response to DNA damage, DNA-PK may repress rRNA transcription by using both direct and indirect pathways.

6.1. Future Perspectives

The finding that the nuclear matrix protein C1D interacts specifically with one of the essential components of the Pol I transcription system no doubt provides evidence that the nuclear matrix plays important roles in transcriptional regulation besides several other

cellular processes. The important task now is to identify the functional and biological significance of this interaction. In other words, the experiments will be organized towards proving or disproving the hypotheses proposed above. One important thing to show will be whether interaction between TAFI 48 and C1D really holds back the formation of SL1 on to the promoter as suggested in the first model. This experiment is rather easy to perform providing that a pure TAFI 110, 63, 48 and TBP are obtained. As mentioned before, it was impossible to express TAFI 48 in bacteria. The open reading frame of TAFI 48 has already been cloned into a baculovirus expression plasmid and studies into expression and purification of TAFI 48 in insect cells should be undertaken in the near future. The other proteins can be expressed and purified in bacteria. Once the pure proteins are obtained, transcription factor SL1 could be reconstituted in the presence and/or absence of C1D to determine whether the reconstituted complexes are as active in supporting transcription from the human ribosomal RNA gene promoter as endogenous SL1. In this context, it would also be possible to perform the same experiments using the *in vitro* phosphorylated C1D.

Another important issue is to demonstrate the role of phosphorylated versus unphosphorylated C1D in Pol I transcription. For this purpose an *in vitro* run on/off assay could be used, ideally using highly purified Pol I basal transcription components. However, in short term, just by using a baculovirus purified TAFI 48 and a TAFI 48 immunodepleted whole cell extract, it would be possible to detect the effects of various forms of C1D on TAFI 48 and as a consequence, on *in vitro* transcription of Pol I. Also the efficiency of transcriptional inhibition by DNA-PK on circular templates in the presence or absence of C1D could be checked with the *in vitro* transcription assays.

In order to obtain more solid evidence about the regulatory role of DNA-PK on C1D/TAFI 48 complex and Pol I transcriptional repression, human cell lines wild type and mutant for DNA-PK could be exposed to γ-irradiation and the interaction between C1D and TAFI 48 and the rRNA levels could be checked. The cells could be synchronized to examine if the regulation is cell cycle dependent. Also in these cells, the colocalization of TAFI 48, C1D and DNA-PK could be checked by immunofluorescence. It will be an idea to knock-out C1D on the wild type and check whether there will be any alterations in the Pol I transcription levels when cells are exposed to γ-irradiation.

In conclusion, in this work, the interaction of two proteins have been verified, and now it is time to find an answer to the questions why and how these proteins interact under biological conditions.

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