CHAPTER - 2

DISCUSSION
Functional significance of the trans-acting proteins in c-jun transcription

Transcriptional regulation of c-jun is highly complex and involves several cis-acting elements and trans-acting proteins that regulate its transcription differentially under different physiological conditions. We have demonstrated the presence of a cis-acting regulatory element spanning -538 to -514 region of c-jun which plays a positive role in c-jun transcription (Agarwal et al., 2008). Knowledge about the proteins that interact with the identified cis-acting element and modulate c-jun expression can be exploited for designing small molecule inhibitors to curtail c-jun expression during pathophysiological conditions. The concept of exploiting transcription factors as potential targets for the development of novel therapeutics is well established. The development of these new generation drugs is based on their ability to modulate either the synthesis of transcription factors or the regulation of their activity (either by phosphorylation or by ligand binding). Thus, an understanding of the mechanism of action of these drugs and the mechanisms of transcriptional regulation offers hope for designing better and promising molecules with higher specificity and fewer side effects (Latchman, 1997; Kletsas and Papavassiliou, 1999; Latchman, 2000).

The strategies employed to target c-Jun to control cell proliferation cannot be exploited due to its involvement in various other biological processes. Therefore, instead of targeting c-Jun, tools can be designed to target the proteins that play a key role in c-jun transcription. Thus, to achieve this, our initial studies on the identification of the positive regulatory cis-element were extended further to the identification of the proteins interacting with it and to decipher the role and mechanism(s) of these proteins in modulating c-jun transcription. In the present chapter, the functional significance of an ≈ 34 kDa protein identified as Translocated promoter region protein (Tpr) and ≈ 45 kDa protein as β-actin in c-jun transcription has been discussed.

The cotransfection experiments with plasmids overexpressing both the proteins, Tpr and β-actin, unequivocally demonstrated the role of Tpr protein in augmenting c-jun transcription. Until recently, role of Tpr has been studied as a component of nuclear pore complex (a nuclear architectural protein, Figure 1). The nuclear pore complex (NPC) constitutes a large organelle, interlinking the inner
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and outer membranes of the nuclear envelope. The presence of these pore complexes at the nuclear periphery facilitates the passive diffusion of ions, metabolites and small molecules across the membrane efficiently. The NPC also regulates the shuttling of the ribonucleoprotein particles from the nucleus to the cytoplasm. Nuclear pore complex proteins are also known to govern the mRNA export post transcription to the cytoplasm for their subsequent translation. The signal mediated transport of macromolecules across the nuclear membrane via the NPC is thus a highly regulated process and points to an important mechanism for control of gene expression (Gerace, 1992; Whiteside and Goodbourn, 1993).

![Figure 1: Nuclear pore complex organization and its components](image)

*Adapted from Bangs et al., 1998; Nakielny and Dreyfuss, 1999*

The actual function of NPC-associated Tpr, occurring ubiquitously in all the vertebrate cells investigated, remained unknown. Studies have suggested that most of the genes (active and inactive) which are localized at the edges of the chromosomal territories provide means of concentrating and coordinating gene transcription, mRNA processing and mRNA transport (Bangs et al., 1998; Nakielny and Dreyfuss, 1999; Skaggs et al., 2007). Several nucleoporins contain typical conserved motifs that provide evidence to their probable functions. Unlike Tpr, Nup153, a nucleoporin contains four zinc finger motifs through which it binds to
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DNA in a zinc dependent fashion in vitro (Pante and Aebi, 1994; Pante et al., 1994; Sukegawa and Blobel, 1993). This is consistent with the proposal that nucleoporins play an important role in the chromatin organization and nucleocytoplasmic transport (Blobel, 1985). Similar to the Tpr, Nup107 also possesses leucine zippers at its C-terminus, suggesting that either dimerization or the formation of homo-or heteromers is a requisite for its functioning (Radu et al., 1995).

The first report on the identification of the Tpr (Translocated promoter region or tumor potentiating region gene sequence, mapped to chromosome 1) in fusion with several proto-oncogenes and the potential of this chimera to induce neoplastic transformation provides an evidence to the importance of chromosomal translocations in rendering normal cells tumorigenic (Yunis, 1983; Weinberg, 1985; Campisi et al., 1984). Such chromosomal translocations and transgenic expression of Tpr (involving $\approx$140-230 NH$_2$ terminal residues) with the protein kinase domains of several proto-oncogenes (ras, raf, met, trc) have been implicated in cellular transformations and hyperplasia (Soman et al., 1991; Liang et al., 1996; Greco et al., 1992). The importance of gene rearrangements in the development of neoplasia makes this transforming sequence, the Tpr (which was identified for the first time from the cell line rendered tumorigenic with the carcinogen, N-methyl N'-nitrosoguanidine, MNNG), important for the present study (Park et al., 1986; Dean et al., 1987; Miranda et al., 1994). The sequence of the human tpr gene from the MNNG-HOS cell line, surrounding the rearrangement revealed that it contained an Alu family repeat (Dean et al., 1987). Alignment of the tpr repeat with the consensus Alu sequence suggest that their lies 84 % homology between the two Alu repeats which are often characterized by long stretches of A residues and the tpr-met rearrangement occurs at the end of the poly(A) stretch following the tpr Alu sequence (Jelinek and Schmid, 1982).

The well characterized break point sequence in the tpr gene thus is responsible for the translocations resulting in cell transformation. Normally by itself Tpr does not possess oncogenic/transforming properties but its interaction with the fusion partners alters its ability to promote cell differentiation and growth. The one
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such characterized fusion of the Tpr is the TPR-MET, a transforming counterpart of the c-MET proto-oncogene detected in human cancer. Therefore, discovery of such genes and their fusion products can be exploited as potential targets for cancer therapy. However, the role of this protein alone in modulating c-jun transcription remains enigmatic. Contrary to this, it has been reported as a tumor suppressor in human gastric cancers (Cunningham et al., 1997; Yu et al., 2000).

Homologs of the Tpr have been identified in both Drosophila melanogaster and Saccharomyces cerevisiae. It has been reported that overexpression of Tpr in mammalian cells and yeast resulted in intranuclear mRNA accumulation thereby inhibiting its subsequent cytoplasmic translocation (Bangs et al., 1998). Consistent with these reports, our study also revealed that CHO-K1 cells overexpressing Tpr underwent apoptosis. Conversely, the two yeast Tpr homologues when deleted in vivo, exploiting gene knockout studies did not affect the cell viability and resulted only in minor or no alterations in nucleocytoplasmic transport (Strambio-de-Castillia et al., 1999). Similarly, the depletion of Tpr pools within the cells using siRNA approach in our studies did not reveal any marked morphological or phenotypical change(s). Therefore, the down-regulation of c-Jun can be successfully exploited for cancer therapy.

Varying lengths of Tpr polypeptides have been characterized in great detail; 265 kDa (Mus musculus), 267 kDa (Homosapiens), 218 kDa (D. melanogaster) to 218 kDa and 195 kDa (S. cerevisiae) (Mitchell and Cooper, 1992a; Zimowska et al., 1997; Strambio-de-Castillia et al., 1999). It has been reported that alternative splicing of human Tpr homolog constituting an ORF of 726 amino acids might result from a deletion of a 30 bp sequence that spans the translation termination site of this ORF, thereby generating mRNAs encoding a tpr protein with an extended C-terminal domain (Mitchell et al., 1992b). The 726 amino acid tpr protein from human is predicted to have extensive regions of alpha-helix and has three stretches of a heptad repeat motif that is characteristic of proteins adopting a coiled-coil conformation. Thus, the Tpr protein from higher order vertebrates represents a conspicuously large protein with an extended C-terminal domain, which is absent from the Tpr homolog identified in our study, which encodes a
small protein of 339 amino acids (BC101883). Thus, it could likely be an alternative splice variant of human Tpr with an altogether different function.

Amino acid sequence analysis (Figure 2) of Rn-Tpr revealed three putative protein kinase C (PKC) phosphorylation sites (Figure 2, shaded grey boxes). Immunoprecipitation experiments of human Tpr from 32P-orthophosphate-labeled cells have demonstrated that the Tpr is a phosphorylated protein of the nuclear interior (Bangs et al., 1996). Thus, phosphorylation of Tpr could be envisioned as one of the mechanisms governing either its trans-activation or DNA-binding. Our earlier observations support this hypothesis wherein we had reported that the trans-acting factor(s) from RNE-d interacted with the cis-element spanning -538 to -514 region of c-jun only in the phosphorylated form (Agarwal et al., 2008). It has also been reported that Tpr-Met chimera possesses an inherent protein kinase activity with the potential to be phosphorylated by Mitogen activated protein kinases (MAPK) (Soman et al., 1991; Greco et al., 1992; Bangs et al., 1996). Our results also supported this fact since the phosphorylation of Tpr in the presence of protein kinase C agonist modulated c-jun transcription significantly. Thus, it could be suggested that the stimulation of c-jun transcription in the CHO-K1 cells transfected with p123jun25b-eGFP plasmid in the presence of TPA might be due to an enhanced phosphorylation of Tpr protein. Hence, the phosphorylation of the

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**Figure 2: Amino acid sequence of the Tpr protein with putative recognition motifs.** The sequence of the 339 amino acids encoding the Tpr protein fragment of *R. novergicus* is shown. The potential leucine zipper motifs at positions 78, 120, 127 and 236 are shown in the blue color and in bold. The putative protein kinase C phosphorylation sites at positions 93, 157 and 175 are in shaded boxes. The amino acid residues constituting the putative Asn glycosylation sites are depicted in bold, italics and underlined. The stretch of the amino acid residues speculated to interact with the Jun-25SA (104D, 106N, 107L, 108G, 109I, 110Q and 112Q) are shown in red as bold.
Tpr prior to its interaction with the chromatin might be an important mechanism governing c-jun trans-activation.

The cis-acting regulatory sequences often contain certain discrete motifs to which specific transcription factor(s) interact. Such DNA sequences induce transcription of genes by extracellular signals such as exposure to heat, heavy metals, viral trans-activators, elevated levels of cAMP and an array of natural and synthetic mitogens. TRE is one such well-characterized cis-acting sequence found in the regulatory regions of many genes which is responsive to TPA (Angel et al., 1987a and b; Lee et al., 1987). Sequence analysis of the 25 bp cis-regulatory element spanning -538 to -514 region of c-jun revealed a TPA like response element (TRE) (TGACTTC) which matched exactly with the consensus TRE like sequence reported earlier (TGAG/CTCA) (Angel and Karin, 1998), except for the replacement of a purine (A) with a pyrimidine (C) at the last position. It has been reported that the proteins that interact with the TRE sequence can tolerate subtle nucleotide variations in TRE and may retain its responsiveness to TPA but can possess different characteristics. TRE’s are usually recognized by either the complex of c-Jun/c-Fos or the proteins with leucine zipper motifs (Bohmann et al., 1987; Angel et al., 1988; Bos et al., 1988; Curran and Franza, 1988; Halazonetis et al., 1988; Kouzarides and Ziff, 1988; Rauscher et al., 1988; Lamph et al., 1988; Schonthal et al., 1988a,b; Cohen et al., 1989; Sassone-Corsi et al., 1990; Masquilier and Sassone-Corsi, 1992; Smith et al., 1993). It has been reported that there is a high degree of sequence similarity of the Tpr with Vimentin, a coiled coil protein with 84 % sequence similarity to Jun, Fos and CREB (Capetanaki et al., 1990).Therefore, this sequence similarity of the Tpr to the c-Jun and Fos points to the possible interaction of the Tpr protein to the TRE like sequence present in the Jun-25sa. The sequence analysis of the Rn-Tpr revealed four potential leucine Zipper motifs (Figure 2, marked in blue) similar to those present in the Jun and Fos, the members of the bZip family of transcription factors (Capetanaki et al., 1990), further strengthened the specific recognition of the Tpr protein to the TRE like sequence in Jun-25sa.
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Direct evidence of the *in vivo* interaction of the Tpr with the chromatin (-538 to -514 region of *c-jun*) and its role in augmenting *c-jun* transcription has been demonstrated. Present investigation has clearly indicated *in vivo* interaction of the Tpr with the chromatin (-538 to -514 region of *c-jun*) and its role in upregulation of *c-jun* transcription. The specific and direct interaction of Tpr to its cognate recognition sequence prompted us to investigate the parameters of Jun-25sA-Tpr interaction in great detail. The observed two fold higher affinity of the rTpr (2 μM) to the 25 bp recognition sequence (Jun-25sA) in comparison to the β-actin (4 μM) was expected and justified considering its role in enhancing *c-jun* transcription. The observed affinity for the rTpr-DNA interaction in micromolar range is in agreement with the affinity reported for the GCN4 homodimers, a DNA-binding protein of bZip family (Berger *et al.*, 1998). The dissociation constants for the interaction of several bZip proteins including the Epstein-barr virus bZIP transcription factor, Zta, Jun-Fos heterodimer, AP-1, C/EBP, XRE1, E-box and HRE to their respective recognition sequences were also reported to be in micronano molar ranges (Doi *et al.*, 2002; Hicks *et al.*, 2003; Fedorova *et al.*, 2006). These results demonstrate that the bZIP proteins are quite versatile with respect to their DNA-binding affinities.

The stoichiometry of Tpr-DNA interaction was established to be 2:1. The existence of dimeric species of the rTpr in solution with a predominance of monomer indicates that the rTpr although exist as a monomer and interact with DNA as a dimer. This is in agreement with the stoichiometry observed with other members of the bZip family of proteins which bind to their cognate recognition sequence either through homo- or hetero-dimerization (Capetanaki *et al.*, 1990; Berger *et al.*, 1998).

Although β-actin was purified along with Tpr in DNA affinity chromatography, transient transfection analysis data clearly indicated that it did not enhance transcription from the *c-jun* promoter by itself. Also, it bound to the Jun-25sA only at 5 fold higher concentration when compared to the Tpr. These data suggest that β-actin might be acting as a mere stabilizing protein. Earlier reports on the nuclear localization of actin were viewed with great uncertainty and
its presence within the nucleus was previously considered to be an "artifact". However, the diverse functions of actin, a traditionally cytoplasmic protein gained evidence recently (Rando et al., 2000; Pederson and Aebi, 2002; Percipalle and Visa, 2006; Grummt, 2006). Its association although with a lesser affinity to the chromatin (-538 to -514 region of c-jun) was in line with the reports suggesting its role in chromatin remodelling (Olave et al., 2002). The direct role of β-actin in transcription as a part of pre-initiation complex with all the three RNA polymerases and its direct binding to the 27 nt repeat sequence to stimulate eNOS expression has already been established (Ou and Shen., 2005; Hoffman et al., 2004; Hu et al., 2004). Thus, the absence of its role in transcription but a modest interaction with the chromatin prompted us to investigate its possible role as a scaffolding protein in stabilizing the Tpr-Jun-25SA complex. This became evident by it's in vivo and in vitro interaction with Tpr. Coimmunoprecipitated Tpr and β-actin from RNE-d when compared with the recombinant proteins revealed a slightly reduced mobility which can be attributed to their post-translational modification under native conditions. This hypothesis holds true since there are several putative N-linked glycosylations sites present in both Tpr and β-actin proteins (Figure 2, bold, italicized and underlined). Co-immunoprecipitation of RNA polymerase II with β-actin has already been established (Hoffman et al., 2004). Also, the association of Tpr with several nucleoporins and other proteins including importin-β has been established (Cordes et al., 1998). However, the interaction of Tpr with actin has not yet been documented. The present study thus reports for the first time the interaction of Tpr and β-actin, to form a functional complex at -538 to -514 region of c-jun. Collectively, the results demonstrated that the interaction of Tpr with the 25 bp cis-element present between -538 to -514 region of the c-jun possibly resulted in loop formation bringing far upstream regions in close proximity to the basal transcription machinery, which can competently drive c-jun transcription, and that the β-actin acted as a stabilizing protein clasping RNA-polymerase II and the basal transcription apparatus together (Hoffman et al., 2004).

The 1:1 stoichiometery of β-actin-Jun-25SA, 2:1 stoichiometery of Tpr-Jun-25SA and 2:1 stoichiometery of Tpr-β-actin interaction provided an insight into the
molecular organization of the ternary complex at the 25 bp cis-acting element. Therefore, based on our results, we generated a hypothetical model demonstrating a functional interaction of Tpr dimers along with β-actin to the chromatin spanning -538 to -514 region of c-jun (Jun-25SA) (Figure 3).

![Figure 3: Hypothetical model depicting the c-jun transcriptional upregulation mediated by the interaction of the dimers of the Tpr with monomeric β-actin at -538 to -514 region of c-jun (Jun-25SA).](image)

Insights into the structural aspects of the Tpr revealed that the rTpr lacked a higher order tertiary structure although possesses a well defined secondary structure. Secondary structure analysis of the rTpr using far-UV CD spectroscopy revealed that the majority of the CD signal obtained for rTpr depicted a negative ellipticity at 208 nm and at 222 nm, suggesting a predominance of α-helix. This is in agreement with the secondary structure values obtained for the human Tpr homologue indicating the predominance of α-helices (Hase et al., 2001). However, little is known about the structure of individual nucleoporins of eukaryotes. Contrary to the significant secondary structure for Rn-Tpr, S. cerevisiae nucleoporin has been characterized as natively unfolded protein with low secondary structure content (Denning et al., 2002). The Tm calculated for the rTpr (≈ 55 °C) by the spectrophotometric analysis, substantiated our earlier findings that suggested that the complex formation between factors present in RNE-d and Jun-25SA is sensitive to the temperatures higher than 45 °C (Agarwal et al., 2008). The absence of complex formation (RNE-d and Jun-25SA) at temperatures higher than 45 °C in an EMSA could be attributed to the partial denaturation of the Tpr in RNE-d (Chapter 1). The Tm calculated for the rTpr fell within the range of Tm
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Approximately, half of the eukaryotic proteins and cancer associated proteins, involved in signalling, regulation contain substantial regions of intrinsic disorder (Campbell et al., 2000; Dunker et al., 2001; Daughdrill et al., 2005; Uversky et al., 2006; Dunker et al., 2008). Involvement of highly flexible proteins in signalling appears logical as a more flexible protein is able to undergo conformational changes in response to environmental perturbations more readily than a rigid one. Thus, the increased disorder in the proteins suggests an increased need for their regulation (Dunker et al., 1997, 1998; Lefstìn and Yamamoto, 1998; Uversky et al., 2000; Demchenko, 2001; Iakoucheva et al., 2002; Kumar et al., 2004; Dyson and Wright, 2005; Liu et al., 2006). These proteins are often called intrinsically disordered proteins and assume either partially or completely folded conformations upon ligand-binding or changes in their microenvironment (Kalodimos et al., 2004; Oldfield et al., 2005; Sharma et al., 2007). The DNA-binding and protein folding has been reported to be coupled for Jun-Fos heterodimeric transcription factor, a bZip protein (Seldeen et al., 2008). This prompted us to evaluate the structure and conformational flexibility of the Rn-Tpr, classified as a bZip protein based on its amino acid sequence and propensity to dimerize in vitro. The ability of the rTpr to interact with ANS that binds to the exposed hydrophobic patches within the protein, confirms that the rTpr is a molten globule with an ordered secondary structure and lacks a well defined higher order tertiary structure. The ability of the Tpr to interact with ANS was almost similar to the ANS binding potential of the clusterin, a protein possessing a substantial molten globule region (Bailey et al., 2001). It is important to note that 32 % decrease in the ANS binding of the rTpr upon DNA addition reflects the formation of a well defined structure following Jun-25SA binding. The rTpr attained a significant tertiary structure upon ligand binding (48 %) as visualized by the near-UV CD spectroscopy and DSC measurements. This gain in the structure of the rTpr was in accordance with the ligand (DNA/protein/ion/small molecule) induced conformation changes in an otherwise premolten globule protein (Horiuchi et al., 1997; Uversky et al., 2000; Bailey et al., 2001; Bell et al.,
Once the DNA induced tertiary structure formation in the rTpr had been established, other possible structure inducers were also tried. Polyols are known to impart structural stability to the proteins (Tiwari and Bhat, 2006). It was observed that sorbitol was also able to provide the rTpr with a respectable tertiary structure. The rTpr in complex with the Jun-25SA depicted a sharp transition while the $T_m$ of the complex slightly increased ($T_m\approx 47$ °C to $52$ °C), suggesting that the interaction of the DNA with the protein does not provide thermal stability to the rTpr. Rather, it provides a well defined tertiary structure necessary for its biological function, which is regulation of gene expression in this case. Apart from a single transition, there is an additional unfolding ($T_m \approx 68$ °C, observed as a small shoulder) in the protein post DNA interaction. This peak in the protein only upon ligand binding could be attributed to the folding of the protein in a way wherein two independent domains are formed that melted differentially. Considering the homogenous protein preparation and absence of any transition in the control sample further strengthens this hypothesis. The possibility of an additional transition contributed by DNA was ruled out as the DNA-DNA baseline was acquired and was corrected from the experimental sample. On the contrary, although a sharp transition was observed with Tpr in sorbitol, the $T_m$ was also enhanced several folds which indicates rigidity in the protein ($T_m \approx 58$ °C). However, the functionality of this structurally rigid protein (rigidity induced by sorbitol) was compromised suggesting that the unfoldedness of Tpr is crucial for it to recognize and bind to its cognate recognition sequence, which is generally true for the disordered proteins (Dunker et al., 2008).

Also, extensive studies provide compelling evidence that flexibility and not mere surface exposure of protease reactive groups, is the major determinant for the possible protease cleavage (Bailey et al., 2001). Thus, a disordered region is expected to undergo digestion; $10^5$ to $10^7$ times faster than an ordered one. Our data on the protease sensitivity for Tpr is in agreement with the study wherein a significant increase in the cleavage rate of F helix of myoglobin after its conversion to apomyoglobin (disordered) has been reported (Fontana et al., 1993, 1997; 2004; Picotti et al., 2004).
Once the role of Tpr as a transcriptional regulator has been demonstrated, its nuclear localization was also ascertained. This is the first report describing the nuclear localization of this Rn-Tpr homologue. The molecular segments involved in mediating nuclear import of the Tpr protein and its association with the NPC are unknown. Secondary structure predictions of the human Tpr revealed that the protein is divided into two different functional domains. The amino terminal domain of protein contains several leucine zipper motifs and clusters of heptad repeats typical of α-helices organized as coiled-coils, suggestive of its possible involvement in Tpr's filament association. The carboxyl terminal domain is rich in acidic amino acid residues that may possibly interact with the basic proteins of the nuclear interior. Domains containing NH₂-terminal truncations do, however, accumulate in nucleoplasm. A COOH-terminal domain of human Tpr localizes to the nucleus and also imparts nuclear localization to GFP suggesting that this domain possesses nuclear localization signal (NLS). However, neither the amino-nor the carboxyl terminal domain contains sequence elements that fit into the consensus sequences of the nuclear localization signals (Boulikas, 1993; Nigg, 1997). Therefore, there is a lack of evidence either to the exact localization or the role of the Tpr protein domains in the nuclear or cytoplasmic localization of its homologs. Our study is the first report describing the nuclear localization of the Rn-Tpr homologue and is in agreement with the previous findings reporting the localization of human and drosophila Tpr homologs within the cytoplasmic side of the NPC and throughout the extra chromosomal nuclear interior, respectively (Byrd et al., 1994; Kosova et al., 2000; Frosst et al., 2002; Krull et al., 2004). The usual large size of NPC polypeptides (>30 kDa) and their inability to passively diffuse through the NPC on their own, points towards existence of a nuclear export mechanism(s) leading to their transport from the site of synthesis. However, analysis of the amino acid sequence of the Rn-Tpr homolog for any possible nuclear localization signal (NLS) revealed absence of any such signal at either the N-or C-termini, suggesting the involvement of a signal independent protein mediated export machinery. Existence of a well defined and established mechanism(s) facilitating the transport of several nuclear pore proteins in a nuclear localization signal (NLS) independent fashion has been documented.
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(Simos and Hurt, 1995; Fagotto et al., 1998). Thus, an alternative mechanism is proposed for the Tpr protein, wherein it may "piggy back" riding its way into the nucleus by interacting with another client protein destined to be transported into the nucleus. Nuclear Tpr protein and other nucleoporins have recently been reported to re-localize to kinetochores during mitosis (Lee et al., 2008). Therefore, the contradictions in the localization of these nuclear pore proteins from pore complexes; nucleolus to chromatin reflects the possibility of the involvement of Tpr in all the three nuclear regions. The possible link between the mRNA transport, regulation of transcription and chromosomal structural maintenance through Tpr points to its diverse functions within the nucleus. Thus, this additional knowledge about the localization and function of this Rn-Tpr homolg in the present study opens up newer avenues to explore into many other dynamic functions of yet other unexplored Tpr homologs. The studies on the role of Tpr homologs either in transcription or any other nuclear process will be of great interest and might give an insight into mechanisms which are yet unveiled.

To elucidate the role of N-terminal and C-terminal region of the rTpr in mediating DNA binding, the deletion fragments of the rTpr were evaluated for their DNA binding ability. It was interesting to note that the N-terminal domain of the protein, possessing three potential leucine zipper motifs was able to interact with the Jun-25sA. Also, the NTD.rTpr was able to interact more efficiently with the DNA in comparison to both the wild type rTpr and the C-terminal domain of the rTpr (CTD.rTpr). This could be attributed to the presence of three zippers in the NTD (in contrast to a sole motif in the CTD), which are known to be involved in protein dimerization and subsequent DNA binding. The role of N-terminal region with potential leucine zippers and DNA binding was in accordance with the DNA binding ability of several transcription factors. It has been reported that the N-terminal half of the α-helix inserts into the minor groove of the DNA, which is the unstructured domain and the other half of the helix, the structured one, is involved in stabilization of the complex, however, the full length protein is required for the desired function (Caroll et al., 1997; Hicks et al., 2003). When NTD.rTpr and CTD.rTpr were reconstituted in vitro, the activity of the reconstituted protein was comparable to the wild type rTpr. The slightly compromised DNA-binding ability of
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the full length rTpr in comparison to the NTD.rTpr, suggested that the presence of both the domains together might stabilize the protein in vivo to drive c-jun transcription. However, the ability of both the domains to transactivate c-jun transcription independently needs to be evaluated to confirm the aforesaid hypothesis.

It is interesting to note that the DNA binding potential of both the halves of the rTpr, reported in the present study was in accordance with their predicted structural analysis. The amino acid sequence constituting both the halves when subjected to the EXPASY proteomics server (http://www.expasy.ch/) for the structure prediction in the protein parameter tool, revealed that the N-terminal of the Tpr is unstructured and unstable when compared to the CTD. This unstructured and unstable nature of NTD substantiated our results and demonstrated the role of this region in DNA binding. Also, the Western blot analysis of the purified NTD.rTpr revealed an additional immunoreactive band at ≈ 38 kDa apart from that obtained at ≈ 18 kDa, pointing towards the ability of the NTD.rTpr to form a dimer in vitro. Thus, the dimerization propensity of the NTD.rTpr might be the plausible reason for its efficient DNA binding ability when compared to the CTD.rTpr.

It has also been documented that the DNA binding region resides in close proximity to the leucine zipper motifs (Carolla et al., 1997). This prompted us to identify the amino acid residues involved in the DNA-protein interaction. The crucial residues in the Tpr required for the DNA binding, based on the homology model were evaluated (Figure 2, depicted in red). The residues crucial for DNA-protein interaction were also localized towards the N-terminal region of the protein. The efficacy of the mutants generated by Site directed mutagenesis was evaluated for their DNA-binding ability. Analysis of the modelled Tpr revealed a glycine at the center of a kink. Glycine and proline are generally considered as helix breakers, and have the propensity to dominate the region with kinks. Therefore, the presence of a kink due to glycine at position 108 was thought to be involved in protein folding to facilitate dimerization and subsequent interaction with the DNA.
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The residues flanking the glycine, were found to be leucine and isoleucine at positions 107 and 109, respectively. The amino acid residues being hydrophobic, were speculated to be involved in base stacking involving hydrophobic interactions. Flanking these hydrophobic residues, the presence of positively charged amino acids, asparagine (N106) and glutamine (Q110) were thought to interact with the negative charged phosphate groups on the Jun-25SA (electrostatic interactions). Therefore, the functional significance of these residues was evaluated by replacing them with alanine. Determination of the DNA binding ability of all the mutants revealed their compromised ability to interact with the DNA in comparison to the wild type rTpr. A significant decrease in the potential of the rTpr.G108A mutant to interact with the DNA might be attributed to its rigidity. The inefficient folding of the glycine mutant was thought to weaken the DNA binding ability by several folds, even though the other residues speculated to be required for DNA binding were intact. Thus, the appropriate flexibility associated with the bZip proteins is critical for their molecular recognition. The mutant Tpr with alanine corresponding to the aspartate and glutamate at positions 104 and 112, respectively, did not show any change in their DNA-binding when compared to the wild type.

Other mutant proteins (rTpr.N106A, rTpr.L107A, rTpr.I109A, rTpr.Q110A) displayed compromised ability to bind to the 25 bp cis-element, even when compared to the rTpr.G108A mutant, suggesting a role for these residues, possibly for either the base stacking (Leucine 107 and Isoleucine 109) or electrostatic interactions mediated by the asparagine at 106th position or the glutamine at 110th position. It is also interesting to note that this cluster of alternate hydrophobic and hydrophilic amino acid residues with glycine at the centre precedes the second leucine zipper motif. Thus, it was confirmed that the DNA binding region resides in the close proximity to the leucine zipper motifs. There are no other glycine centres with such alternate hydrophobic and hydrophilic residues in the overall protein sequence (339 amino acids). Also, the human Tpr sequence (726 amino acids), when aligned with the R. novergicus Tpr using ClustalW,
revealed that the glycine at 108th position of *R. novergicus* Tpr was replaced with alanine in human Tpr homolog (Figure S6). This suggests the possibility of involvement of different set of glycine/proline in the human Tpr to attain similar folding and thus the required biological function, which is not yet explored.

The lack of the information on the proteins with sequence or structural similarities to the Tpr in the Protein database (PDB) rendered the homology modelling of the full length protein unsuccessful. Since the PDB does not contain any structure or relevant information neither for this Tpr homolog nor for its human counterpart, it would be interesting to look into the structural aspects of this entity. Therefore, further studies are in progress to crystallize the protein alone and in conjunction with its ligand, DNA (Jun-25S$_A$) to evaluate the structure of the protein in great detail.

**Future research directions**

The major challenge in the development of a potential drug against cancer primarily involves the identification of putative therapeutic targets. The most potential ones to be exploited include cellular proto-oncogenes that are implicated in tumorigenesis. However, their ability to control vital biological processes renders them ineffective to be used as suitable targets. Thus, the futuristic approach is to identify the proteins that regulate the activity of these proto-oncogenes and may serve as ingenious candidates. The present study identified a transcription factor, Tpr which plays a positive role in *c-jun* transcription and the structural and functional aspects governing this trans-activation has been studied. Studies on the bZip proteins revealed that dimerization is necessary for their function. Therefore, small molecules that could inhibit the dimerization of the transcription factors can be exploited for drug design (Figure 4) (Ferre-D'Amaré *et al.*, 1993; Hicks *et al.*, 2003; Kiessling *et al.*, 2006; Berg, 2008).
Since Tpr is shown to bind to its recognition sequence (-538 to -514 region of the c-jun) as a dimer, small molecules, that would inhibit Tpr dimerization, and its subsequent DNA binding might render it inactive, can be used to target Tpr dimerization. Also, since its interaction with β-actin is necessary for bringing it to the close proximity of the initiation complex, altering chromatin conformation, and blocking of that interaction may also result in the inhibition of the Tpr-mediated c-jun transcription. Identification of such sites in Tpr is necessary to block its interaction with β-actin. The prospects of small molecule analogs in disrupting protein-protein interaction and their subsequent use as novel cancer therapeutics became evident when the MDM2-p53 interaction was specifically blocked by employing site-specific drug, which resulted in stabilization of p53 and subsequent cell cycle arrest in rapidly proliferating cancer cell population (Vassilev, 2004; Vassilev et al., 2004). Also, the recent report on the potential of the intrinsically unstructured proteins as targets for drug discovery opens up newer avenues to carry out studies further in this direction (Salma et al., 2009). Identification of Tpr as a regulatory molecule in c-jun transcription in the present study thus present a new possibility for the development of molecules/structural analogs that may in part downregulate the c-jun transcription. Also, the novel cancer therapeutics that will be generated by exploiting the aforesaid strategy can possibly be used alone or in adjunct with the other existing therapies to inhibit the proliferation of the cancerous cells.