ROLE OF TRANSLOCATED PROMOTER REGION PROTEIN IN TRANSCRIPTIONAL REGULATION OF C-JUN

SUMMARY
The results obtained in the present study are summarized as follows:

**Identification of the cis-acting element involved in the positive regulation of c-jun**

- Transient transfection analysis of the plasmid harboring the -563 to -273 region of c-jun established the functional significance of this region in the positive regulation of c-jun.

- Transient transfection analysis employing several deletion constructs, revealed the role of a 25 bp cis-acting element, spanning -538 to -514 region of c-jun in its positive regulation.

- The positive regulation of c-jun mediated by the 25 bp element spanning -538 to -514 region of c-jun was found to be more pronounced when compared to that obtained with the -148 to -124 region of c-jun (which was established previously in our laboratory).

- The identified 25 bp positive regulatory element modulated the overall c-jun transcription, since the -538 to -514 region of c-jun was able to augment c-jun transcription from both the total c-jun promoter and from the basal promoter.

- Establishment of the role of the positive regulatory cis-elements spanning -538 to -514 and -148 to -124 region of c-jun in conjunction with each other in c-jun transcription revealed that the two elements do not act synergistically and the effect brought about by the -538 to -514 region remained dominant.

- The cis-acting element spanning -538 to -514 region of c-jun (Jun-25\textsubscript{SA}) interacted specifically and with high affinity with the nuclear proteins harvested from the normal rat liver (nRNE-d).

- The EMSA performed with the 5' and 3' end deletion variants of the Jun-25\textsubscript{SA} and RNE-d revealed that the DNA-protein interaction was mediated throughout the 25 bp region.

- The monovalent ions were absolutely essential for the complex formation with an optimum of 100 mM NaCl required for the DNA-protein adduct formation. The complex formation was not dependent on the divalent cations but their presence in some way stabilizes the complex formation.
The protein factor(s) that bind to the Jun-25SA are temperature sensitive and bind to the recognition sequence only in the phosphorylated form.

The protein factor(s) interact with the Jun-25SA through minor groove.

South-Western blot analysis revealed that the -538 to -514 region of c-jun was specifically recognized by the proteins of ≈ 45 kDa and ≈ 34 kDa.

UV-cross linking analysis revealed the formation of three complexes at ≈ 80 kDa, ≈ 45 kDa and ≈ 34 kDa following UV irradiation.

EMSA carried out with the nuclear extracts prepared from normal and regenerating liver revealed that the -538 to -514 region involved in the positive regulation of c-jun was not differentially recognized by the factors present in the quiescent and proliferating rat liver.

The protein factors corresponding to the ≈ 45 kDa and ≈ 34 kDa, interacting with the -538 to -514 region of c-jun were purified to almost homogeneity by DNA-affinity chromatography.

SDS-PAGE analysis of the affinity purified fractions showed two proteins of ≈ 45 kDa and ≈ 34 kDa, eluted at 0.5 M-0.7 M NaCl.

MALDI-TOF-MS analysis revealed the ≈ 45 kDa to be β-actin and the ≈ 34 kDa protein to be Translocated promoter region protein (Tpr) of Rattus norvegicus.

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

Cloning, expression and purification of the recombinant Tpr (rTpr), its N-and C-terminal deletion variants (rTpr.NTD and rTpr.CTD) and β-actin (rβ-actin) of R. norvegicus was accomplished in E. coli.

Cotransfection analysis of the CHO-K1 cells with plasmids overexpressing either the Tpr or β-actin, along with the plasmid harboring the 25 bp cis-element in the GFP plasmid, revealed the role of Tpr in augmenting c-jun transcription and the β-actin had no effect on c-jun transcription.

Cotransfection of CHO-K1 cells with the plasmid encoding siRNA designed against Tpr provided direct evidence to the role of Tpr in c-jun transcription.

The semi-quantitative RT-PCR analysis of the CHO-K1 cells overexpressing the Tpr revealed an enhancement in the c-jun mRNA levels. The level of the c-Jun protein was also enhanced in the cells overexpressing Tpr as assessed by Western blot analysis.
Summary

- The trans-activation mediated by the 25 bp cis-element in the presence of protein kinase C agonist and antagonist revealed the role of phosphorylation of the protein factor(s) involved in the positive regulation of c-jun.

- Chromatin immunoprecipitation revealed that the Tpr and β-actin bind to the specific region (-538 to -514 region of the c-jun) of the chromatin in vivo, although their ability to interact with the chromatin differs; with Tpr interacting with the Jun-25SA with a higher affinity in comparison to the β-actin.

- Immunofluorescence analysis of the HeLa cells and immunoblotting analysis of the CHO-K1 cells revealed that the Rn-Tpr homolog resides within the nucleus of the mammalian cells.

- In vivo interaction of the Tpr and β-actin with each other was established by the co-immunoprecipitation analysis of the RNE-d fraction. The in vitro interaction employing the purified recombinant proteins was confirmed by ELISA and Far-Western blotting.

- The stoichiometry of the interaction of the rTpr and rβ-actin using fluorescence polarization revealed that the 2 molecules of the rTpr binds to 1 molecule of the rβ-actin.

- The rTpr binds more efficiently to the Jun-25SA when compared to the rβ-actin as established by the EtBr displacement assay and EMSA using purified recombinant proteins.

- The dissociation constants for the rTpr-Jun-25SA and rβ-actin-Jun-25SA interaction were calculated to be $2 \times 10^6 \text{ M}^{-1}$ and $4.2 \times 10^6 \text{ M}^{-1}$, respectively, using fluorescence spectroscopy.

- Determination of the stoichiometry for the rTpr and rβ-actin interaction with the Jun-25SA revealed that the one molecule of the Jun-25SA interacted with two molecules of the rTpr and one molecule of the rβ-actin, respectively.

- The rTpr has the propensity to form dimers, which are known to interact with the Jun-25SA, however, it exists predominantly as a monomer, as established by UV and chemical cross-linking experiments.

- The rTpr is a molten globule like protein possessing a well defined secondary structure and lacks a higher order tertiary structure as assessed by the far-UV and near-UV CD spectroscopic analysis, respectively.
ANS binding of the rTpr alone and in conjunction with the Jun-25\textsubscript{SA} revealed that the rTpr attains tertiary structure only upon DNA binding. These results were also substantiated by the DSC analysis. The sensitivity of the rTpr to the protease treatment reflected the structural flexibility associated with the protein.

The Tpr was modelled using 3-D Jigsaw software. The modelled structure revealed that the protein comprised predominantly of the alpha helices with no significant tertiary structure.

The N-terminal domain of the rTpr was implicated in the DNA binding by virtue of its ability to dimerize and owing to its unstable/unstructured region, in comparison to the C-terminal region of the Tpr.

Site directed mutagenesis of the N-terminal amino acid residues of the Tpr; Asparagine (N), Leucine (L), Glycine (G), Isoleucine (I) and Glutamine (Q) at positions 104, 106, 107, 108, 109 and 110, respectively, established their role in DNA binding.