J. Biochem. 2008;144(6)741–752
doi:10.1093/jb/mvn129

Identification and Characterization of a Positive Regulatory *cis*-element Within the Upstream Region of *c-jun*

Shivani Agarwal, Raj Kumar*, Preeti Gupta and Aparna Dixit†

Gene Regulation Laboratory, School of Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India

Received August 31, 2008; accepted September 15, 2008; published online October 3, 2008

Jun oncoprotein, a component of transcription factor AP-1, is responsible for expression of multiple genes which trigger cell proliferation, differentiation and apoptosis. The diverse regulatory roles that c-Jun plays point towards its varied and complex transcriptional regulation under different physiological conditions. This could possibly involve an interplay of different *trans*-acting factors and multiple *cis*-acting elements present in the *c-jun*. It is, therefore, important to identify such regulatory elements that could be involved in its stringent up- or down-regulation. In the present study, transient transfection analysis with stepwise deletion variants of the upstream region (−563 to −273), linked to basal *c-jun* promoter in a reporter plasmid revealed the existence of multiple regulatory regions that modulated gene expression. Further delineation of the region, conferring positive regulation of transcription from the *c-jun* promoter, led to the identification of a *cis*-acting element spanning −538 to −514 region. The *trans*-acting factor(s) present in rat liver nuclear extract interacted specifically only in the phosphorylated form, and with a high affinity to the recognition sequence. UV cross-linking and South-western blotting, in conjunction with the analysis of affinity purified proteins interacting with this region, revealed that at least two protein factors of ∼45 kDa and ∼34 kDa bind specifically to this region.

Key words: *c-jun*, *cis*-acting element, EMSA, positive regulatory factor, transcriptional regulation.

Abbreviations: AP-1, activator protein-1; RNE-d, rat liver nuclear extract fraction-d; EMSA, electrophoretic mobility shift assay; SS-DNA, salmon sperm DNA; CIAP, calf intestinal alkaline phosphatase; RLjunRP, rat liver jun regulatory protein.

Regulation of activities of different transcription factors is essential for appropriate temporal and spatial patterns of gene expression. Constitutive expression of endogenous nuclear oncoprotein products has the potential to induce neoplastic transformation. The c-Jun, an immediate early gene product is a component of sequence-specific AP-1 transcription factor and a molecular integrator of several signal transduction pathways (*1–3*). Cellular expression of this protein is significantly altered in response to a variety of external stimuli including phorbol ester tumour promoters, growth factors, cytokines and UV irradiations. The c-Jun being a component of AP-1 also elicits a wide variety of biological activities depending on the cellular background and has been implicated in the control of differentiation, apoptosis and oncogenesis (*4–6*).

The role of *c-jun* in tumourigenesis has been primarily attributed to the aberrant expression of multiple genes under its control (*7*). Identification of genes down-regulated in jun-transformed fibroblasts has unraveled direct molecular targets of Jun/AP-1 complex. Differential expression of the target genes of c-Jun thereof leads to the conversion of a normal fibroblast into a transformed phenotype (*8–11*). Enhanced transcription of *c-jun* and its translation have been reported to correlate well with the state of cell differentiation in retinoic acid-treated teratocarcinoma cell line (*12*). Also, the alteration in the *c-jun* mRNA expression upon differentiation of acute myeloid leukaemic HL60 cells is lineage specific, as its expression increased upon differentiation of these cells to macrophages but not to granulocytes (*13*, *14*).

Transcriptional regulation of *c-jun* represents a highly complex and important nodal point for diverse cellular signaling pathways. The downstream effectors playing a key role in c-Jun induced signal transduction are quite well documented; however, the exact mechanism(s) underlying its own expression during various cellular processes are poorly understood. Positive auto-regulation of *c-jun* by AP-1 is well established (*15*). The feedback mechanisms that up-regulate its own transcription, is facilitated by overexpression of c-Jun N-terminal kinase (JNK) (*16*). It has also been documented that sites further upstream of the AP-1 have a role in the transcriptional regulation of *c-jun* (*17*, *18*). The studies on the activation of *c-jun* in response to external stimuli via involvement of functional AP-1 sites have uncovered a complex network of protein–DNA interactions; however,

†To whom correspondence should be addressed. Tel: +91-11-26742164/26704085, Fax: +91-11-26717561, E-mail: adix2100@mail.jnu.ac.in; adixit7@yahoo.com; adixit7@hotmail.com
*Present address: Raj Kumar, Division of Health and Reproductive Sciences, Lexington, KY 40202, USA

the role of upstream sequences in the regulation of *c-jun* transcription is yet unexplored. Role of preformed complexes in the upstream regions of the *c-jun* responsible for UV-induced *c-jun* expression have also been reported (*19*). A positive regulatory factor from rat liver, interacting with the −148 to −124 region of *c-jun*, involved in the differential regulation of *c-jun* transcription under different physiological conditions, has earlier been reported (*20*, *21*).

The formation of protein complexes and their interaction with DNA influence the transcriptional machinery that enables specific expression of genes at a precise time and in response to appropriate stimuli. An emerging paradigm suggests that variety of mechanisms exist that allow individual *cis*-elements to interact with a specific *trans*-acting factor(s) under different physiological conditions. This preliminary interaction acts as a nucleation site to which a series of accessory proteins are recruited, and regulate transcription. Considering the variety of roles the c-Jun protein plays, an intricate and complex regulation of its transcription in different cellular processes is expected. Till date, a number of *cis*-acting regulatory sites up to 200 bp upstream of the transcription start site in the *c-jun* have been identified. However, the possibility of other regulatory molecular switches that could be turned on/off under variety of different physiological conditions, involving multiple *cis*-acting elements that are recognized by myriad of *trans*-acting factors, located further upstream cannot be ruled out. It is likely that the *trans*-acting factors that interact with yet unidentified regulatory sites located further upstream of the *c-jun* promoter are targeted by different signaling molecules under varied physiological conditions/stimuli. Therefore, in order to identify other molecular switches that could regulate *c-jun* expression, upstream region of *c-jun* was analysed for its transcriptional regulatory activity. This resulted in identification of yet another *cis*-acting element located between −538 and −514 region of *c-jun* that up-regulates its transcription.

MATERIALS AND METHODS

Bacterial Strains, Animals, Plasmids, Reagents and Chemicals—Escherichia coli DH5α strain was obtained from GIBCO-BRL. Plasmid −1100/+170jun-CAT was a kind gift from Dr Peter Angel, Institute for Genetik, Kernforschungszentrum Karlsruhe, GmBH Postfach 3640 D-76021, Karlsruhe, Germany. Healthy Wistar strain rats weighing 150–170 g were maintained at the Animal Facility of Jawaharlal Nehru University, New Delhi, India. Animals were fed standard rat chow *ad libitum*. The guidelines prescribed by the Institutional Animal Ethics Committee, JNU, New Delhi, India were followed while handling animals.

All the chemicals used in the study were of analytical grade and were from Sigma Chemical Co., USA unless stated otherwise. The reagents required for DNA manipulation were procured from New England Biolabs, USA or Promega Corporation, USA. The radioactive nucleotide α-^{32}P[dCTP], used in the study was from Board of Radiation and Isotope Technology, Hyderabad, India. DNA manipulations were carried out using standard protocols unless otherwise stated (*22*). Oligonucleotides and primers used in the study were synthesized by Microsynth, Switzerland.

Fractionation of Nuclear Extract—Animals were sacrificed by cervical dislocation and the extracted liver was washed in chilled saline and nuclear extract was prepared as described (*23*). Partial hepatectomy (70%) was performed on animals as described (*24*). The fraction designated RNE-d consisting of maximum RNA polymerase II activity and vital transcription factors was used in electrophoretic mobility shift assay (EMSA) and other studies.

Transient Transfection and Reporter Gene Assay—Construction of reporter plasmids

The transcription efficiency of various plasmids harbouring upstream regions of *c-jun* was assessed by monitoring the reporter gene (GFP) expression in transient transfection assay (*25*, *26*). To identify the regulatory sequences present within −563 to −273 region of the *c-jun*, several fragments harbouring 5′- end deletions of this region were generated by PCR using plasmid −1100/+170 jun-CAT as a template using region specific primers and Vent DNA polymerase (Promega, USA). The PCR amplified fragments were cloned in the end-filled *Sal*I digested plasmid p123*jun*-eGFP upstream of the basal *c-jun* promoter (*20*). The transformants were screened for the presence of the insert by restriction enzyme digestion and confirmed by DNA sequencing analysis.

In order to evaluate the potential of −538 to −514 region in modulating total *c-jun* promoter activity, reporter constructs harbouring −538 to +53 and −514 to +53 region of *c-jun* were made. For this purpose, the respective fragments were generated by PCR amplification using region-specific primers and the plasmid −1100/+170 jun-CAT as a template. The *Ase*I and *EcoR*I sites were incorporated in the forward and reverse primers for convenient cloning. The PCR amplified fragments, digested with the *Ase*I and *Eco*RI sites, were cloned in *Ase*I and *EcoR*I digested peGFP-N1 vector (Clontech, Ltd). Cloning of the promoter fragments in pEGFP-N1 vector with these enzymes resulted in the replacement of CMV promoter with the *c-jun* promoter fragments, thereby placing GFP under their control. Putative recombinants were confirmed by *Ase*I and *Eco*RI digestion followed by DNA sequencing. The constructs were designated as p538*jun*-eGFP and p514*jun*-eGFP.

A positive regulatory element, spanning −148 to −124 region of *c-jun*, had earlier been identified in our laboratory (*20*). In order to assess if the positive regulatory element (Jun-25$_{SA}$ spanning −538 to −514 region) identified in the present study, exert a synergistic or additive effect on *c-jun* transcription, oligonucleotides encompassing the −538 to −514 region was cloned in the *Sal*I digested p148*jun*-eGFP. The transformants were confirmed for the presence and the orientation of the insert by *Apa*I digestion and DNA sequencing. The promoter chimeric construct, thus constructed, was designated as p148*jun*25b-eGFP. Plasmid DNA for transfection assays was prepared using Qiagen DNA preparation kit from Qiagen, Germany, as per the manufacturer's instructions.

Transient transfection analysis

CHO cells used for the transfection were maintained in RPMI (Biological Industries, Israel) supplemented with 10% fetal calf serum (heat inactivated), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Transfection of CHO cells with the reporter plasmid constructs was carried out using lipofectamine reagent essentially as described earlier. The cells were transfected with equimolar concentrations of the test plasmids similarly to the 2.5 μg of control plasmid and 5 μg of the lipofectamine reagent. One microgram of pSV40-β-gal plasmid was co-transfected with the test plasmids. The cells were lysed 48 h post-transfection and the lysates were analysed for GFP expression and β-galactosidase activity as described (*20*). The difference in the transfection efficiency between different samples is normalized by taking the ratio of the observed GFP fluorescence and β-galactosidase activity in the respective sample. The results are reported as the ratio of the observed fluorescence to β-galactosidase activity in the respective sample. To assess the effect of different upstream region of *c-jun* on the *c-jun* promoter activity, the expression level obtained with the control plasmid p123*jun*-eGFP, harbouring only the basal promoter of *c-jun*, was taken as 100%. The reported results are the average of three independent transfections performed in triplicates.

Electrophoretic Mobility Shift Assay—The sequence of the DNA element, identified after transfection analysis was chemically synthesized from Microsynth, Switzerland. The two complimentary oligonucleotides, spanning −538 to −514 region of *c-jun* [(A) 5′-GGATGAC TTCGGGCCC-3′ and (B) 5′-GGGCCCGAAGTCATCCACC GCGGCC-3′ were designed in a way to leave an overhang of eight bases upon annealing] were annealed and radiolabelled. Radiolabelling of the annealed oligonucletides with α-^{32}P[dCTP] by end-filling using Klenow fragment resulted in a 25 bp long oligonucleotide and therefore, is referred to as Jun-25_{SA} henceforth. EMSA was carried out using fraction RNE-d and rRNE-d and radiolabelled Jun-25_{SA} as described with minor modifications (*20*). Varying concentrations of RNE-d [pre-incubated with 500 ng of non-specific salmon sperm DNA (SS-DNA) for 15 min] were incubated with 2 ng of labelled Jun-25_{SA} (∼ 40,000 c.p.m.) in a reaction mixture containing binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 1.0 mM EDTA, 0.1% Triton X-100, 5% glycerol) in a final reaction volume of 40 μl at 30°C for 30 min, unless stated otherwise. The complex was loaded onto a pre-electrophoresed 5% non-denaturing PAGE in Tris–Glycine buffer (0.192 M Glycine, 25 mM Tris–HCl, pH 8.3) at 11 V/cm. For competition experiments, the RNE-d was pre-incubated with unlabelled Jun-25_{SA} or fragmented non-specific SS-DNA prior to the addition of labelled Jun-25_{SA}. The products were analysed by autoradiography.

UV Cross-linking Analysis—To determine the approximate molecular mass of protein–DNA complexes, UV cross-linking techniques are routinely employed (*27–29*). The EMSA reaction was carried out for 30 min using 5 ng of labelled oligonucleotide, Jun-25_{SA} and 100–200 μg of RNE-d as described (*20*). The reaction mixture was placed on ice and UV-irradiated (254 nm) for 15 min. Following cross-linking, the mixture was separated on 12% SDS–PAGE and analysed by autoradiography.

South-Western Blotting—South-western blotting was performed essentially as described earlier (*20*, *30*). Briefly, 50 μg of RNE-d was fractionated on 12% SDS–PAGE and transblotted onto nitrocellulose membrane. The blot was pre-incubated with the blocking solution containing non-specific DNA, prior to incubation with the radiolabelled Jun-25_{SA} as a probe and analysed by autoradiography. The protein factor(s) that are detected by autoradiography depict the specific DNA-binding transcription factor(s).

Affinity Purification of the trans-acting Factors Interacting with −538 to −514 Region of c-jun—The purification of the *trans*-acting factor(s), specifically interacting with Jun-25_{SA} was carried out using DNA affinity chromatography using cyanogen bromide- activated sepharose (CNBr-activated sepharose) as described by Kadonaga and Tijan (*31*). The DNA was coupled to commercially available CNBr-activated sepharose CL-4B resin and the purification steps were followed as described (*20*, *21*). The bound protein(s) were eluted with binding buffer containing a linear gradient of NaCl. The presence of protein(s) in the eluted fractions was analysed spectrophotometrically at 280 nm. The protein fractions were frozen rapidly in liquid nitrogen and stored at −70°C till further use. Aliquots of various fractions were analysed for their ability to bind to DNA using EMSA, UV cross-linking and South-western blotting. The protein fraction(s) with the DNA binding potential were analysed on 12% SDS–PAGE.

RESULTS AND DISCUSSION

The −563 to −273 Region of c-jun Positively Regulates Transcription from c-jun Promoter—To characterize the transcriptional regulation of *c-jun*, we sought to map the far upstream regions that modulate the promoter activity. Transcriptional initiation of *c-jun* from the core promoter has already been established but the molecular interactions that occur between the DNA-binding factors and components of the basal transcription machinery and other accessory transcription factors remain elusive. Earlier studies have suggested that binding of AP-1 to its consensus sequence significantly enhanced *c-jun* expression (*2*). Identification of a positive regulatory element in the upstream region of *c-jun*, spanning −148 to −124 region and its role in response to growth and proliferation have given an insight into the mechanism(s) underlying *c-jun* transcription (*20*, *21*). Sites further upstream of AP-1 have also been reported to play a positive role in UV-induced expression of *c-jun*. *In vivo* genomic foot printing analysis in the UV-irradiated and TPA induced HeLa cells have led to the identification of regulatory elements that are involved in up-regulation of *c-jun* by these stimuli. However, such elements have been identified up to −191 bp upstream of the transcription initiation site. These investigations established the role of preformed complexes in the upstream region of *c-jun* that are targeted by the signal transduction cascade in response to UV irradiation and TPA treatment (*17–19*, *32*). Considering the diverse cellular processes

in which c-Jun is involved and the complexity associated with the *c-jun* expression and regulation, its transcription regulation possibly involves multiple regulatory elements and potential transcription factor binding sites. The present study was undertaken to delineate the additional regulatory sequences present upstream of this region that may have a plausible role in *c-jun* transcription. Transient transfection analysis of the reporter construct harbouring −563 to −273 region was carried out, for assessing its role in transcription, if any. For this purpose, the DNA fragment spanning −563 to −273 region of *c-jun* was cloned upstream of the *c-jun* promoter in plasmid p123*jun*-eGFP. The construct thus generated was designated as p291.123*jun*-eGFP. It was expected that GFP expression from the basal promoter will be altered depending on the presence of regulatory sequence(s) in this region. Transient transfection analysis using this construct (p291.123*jun*-eGFP) revealed that the transcription efficiency of the promoter was enhanced by ∼120%, when compared to the control plasmid p123*jun*-eGFP harbouring the basal promoter only (Fig. 1). The GFP expression in the cells transfected with plasmid p291.123*jun*-eGFP was far greater than that observed in the cells transfected with plasmid p148*jun*-eGFP (∼45%), consisting positive regulatory element (−148 to −124 region cloned upstream of the basal *c-jun* promoter) identified earlier (*20*). These data thus suggest that the −563 to −273 region of *c-jun* is able to effectively augment transcription from *c-jun* basal promoter.

Transcriptional Regulation of c-jun is Multipartite and Depends on the Coordinated Interaction of Several cis-acting Elements and trans-acting Factors—Since the 291 bp region spanning −563 to −273 region of *c-jun* is rather large, attempts were made to further narrow down the boundaries of the regulatory sequences that are responsible for enhanced transcription from *c-jun* promoter. Fragments with 50 and 100 bp deletions from the 5′-end of the 291 bp were cloned upstream of the *c-jun* promoter in plasmid p123*jun*-eGFP. These constructs along with the reference plasmids, p123*jun*-eGFP and p291.123*jun*-eGFP, were assayed for reporter gene expression in a transient transfection analysis. Quantification of GFP, as assessed by the fluorescence measurements, gave a clear indication of the effect of deletions harboured in the parent plasmid. As evident from the relative fluorescence observed with different constructs, each 50 bp deletion in the 291 bp region from the 5′-end affected the GFP expression differentially (Fig. 1), and thus resulted in the identification of more than one regulatory *cis*-acting elements.

As evident from Fig. 1, a deletion of 50 bp from the 5′-end in the −563 to −273 region (plasmid p291$_{\triangle 50}$123*jun*-eGFP) resulted in a drastic decrease (∼50%) in the GFP expression, suggesting the presence of at least one positive regulatory element within this region spanning −563 to −514. Since the extent of stimulation observed with this fragment is ∼50% of that obtained with the wild-type fragment and the GFP expression still remained greater than that observed with the control plasmid p123*jun*-eGFP, it is plausible that additional regulatory sequences may be present in the remaining 241 bp region, i.e. spanning −513 to −273 bp region. The sequence analysis of −563 to −273 region of *c-jun* gene revealed the presence of a sequence GTGGAAAC (between − 508 and −501), which matches with the consensus sequence GTGG(AAA/TTT)G present in viral and some cellular enhancers, with just one guanine at the eighth position being replaced by cytosine (*33–35*). However, further deletion of the 50 bp from the 5′-end (plasmid p291$_{\triangle 100}$123*jun*-eGFP), did not alter the GFP expression significantly, suggesting that the putative enhancer like sequence, GTGGAAAC, present between −508 and −501 may function cooperatively with other sequences within the 291 bp fragment to drive the expression of *c-jun* from the promoter. The presence of additional regulatory sequences between −463 and −273 region can not be ruled out as the plasmid p291$_{\triangle 100}$123*jun*-eGFP in which this region was cloned upstream of the basal *c-jun* promoter still exhibited much higher GFP expression (∼58%) than that observed with the control plasmid.

Delineation of the Positive Regulatory Element Within −563 to −514 Region of c-jun—The region spanning −563 to −514 appears to be important in *c-jun* transcription as its deletion resulted in an ∼50% reduction in the GFP expression in comparison to the plasmid

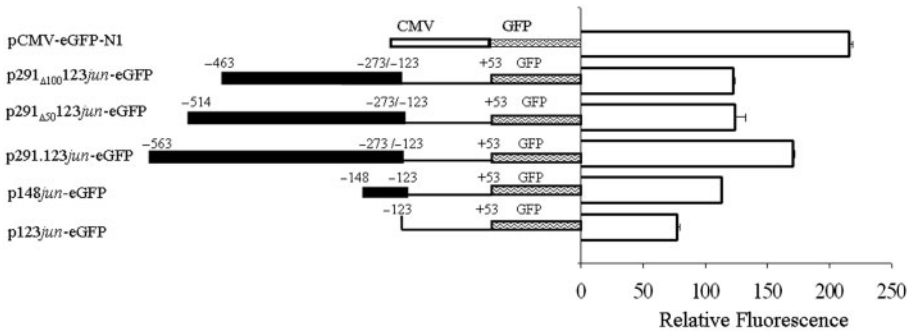

Fig. 1. **Effect of −563 to −273 region and its deletion variants on *c-jun* promoter activity.** Schematic representations of various plasmids used in the transfection assay are given on the left. The plasmid drawings are not to the scale. Plasmids pCMV-GFP and p148*jun*-eGFP plasmid were used as positive controls. CHO cells were transfected with equimolar concentrations of different plasmids along with 1 μg of pSV-β-gal. The data represent the ratio of the observed fluorescence to β-galactosidase activity in the respective samples. Relative fluorescence represents mean ± SD of three independent transfections performed in triplicates with the respective plasmids.

p291.123*jun*-eGFP harbouring the 291 bp fragment spanning −563 to −273 region of the *c-jun* (Fig. 1). In order to confirm the positive regulatory role of this region, a synthetic oligonucleotide corresponding to this region was cloned upstream of the basal *c-jun* promoter in the GFP expression plasmid (p123*jun*50-eGFP). Plasmid p123*jun*50-eGFP showed a significant enhancement (∼80%) in the GFP expression when compared to the control plasmid p123*jun*-eGFP, thus confirming the positive role of this region in *c-jun* transcription.

To further delineate the positive regulatory element present within the 50 bp region spanning −563 to −514 region of the *c-jun*, two oligonucleotides spanning (i) the −538 to −514 region and (ii) the −563 to −539 region were cloned upstream of *c-jun* promoter in plasmid p123,*jun*-eGFP. When an oligonucleotide encompassing only the 25 bp from the 5′-end of the 50 bp positive regulatory region (−563 to −539) was cloned upstream of the *c-jun* basal promoter (plasmid p123*jun*25a-eGFP), no change in the GFP expression was observed in comparison to the basal promoter (Fig. 2). It is possible that part of the positive regulatory element was not included in the 25 bp oligonucleotide used in this clone or the regulatory activity simply does not reside in this region. However, the expression analysis of the clone harbouring the −538 to −514 region upstream of the *c-jun* promoter (p123*jun*25b-eGFP) showed ∼76% enhancement in GFP expression, almost equivalent to that achieved with the clone harbouring the entire 50 bp element harbouring the −563 to −514 region (p123*jun*50-eGFP). The data clearly suggest that the −538 to −514 region itself is able to confer positive regulatory effect on transcription from *c-jun* promoter.

Collective analysis of the data obtained from transfection experiments suggests that the constructs p123*jun*25b-eGFP (harbouring −538 to −514) and p291*jun*-eGFP (harbouring the entire 291 bp region, i.e. −563 to −273) show ∼80% and ∼120% increase in GFP expression when compared to the basal promoter (Figs 1 and 2). Thus, ∼75% of the stimulatory effect brought about by the entire 291 bp region appears to be conferred by the *cis*-acting element(s) present between position −538 and −514, suggesting the presence of strong positive regulatory sequences in this region. Short stretches of DNA possessing enhancer/activator like activity have earlier been reported in a number of genes (*36*, *37*).

In order to assess the effect of the −538 to −514 region on the total *c-jun* promoter activity, transient transfection analysis was carried out using the reporter constructs, harbouring −538 to +53 region of *c-jun* (p538*jun*-eGFP) and −513 to +53 region (p514jun-eGFP) of *c-jun* cloned upstream of the reporter gene. As evident from the Fig. 3, plasmid p538*jun*-eGFP gave ∼36% greater expression of the reporter gene in comparison to that observed with the p514*jun*-eGFP. This clearly indicates that −538 to −514 region is indeed a positive regulator, and able to augment transcription from both the total promoter (ref. plasmid p538*jun*-eGFP) and from the basal promoter (ref. plasmid p123*jun*-eGFP). Differential enhancement in the expression of the reporter gene by −538 to −514 (*jun*25b), when cloned alone upstream of the basal promoter (∼80%) or when present upstream of the whole region (∼36%) can be attributed to the additional positive regulatory elements (such as AP-1 like sequences, NF-jun binding site, CCAAT box element, SP-1 sequences) present in the whole *c-jun* promoter.

The positive regulatory element (−538 to −514) identified in the present study shows higher *trans*-activation potential in comparison to the positive regulatory element located between −148 and −124 region of the *c-jun*, identified earlier (*20*). In order to establish if the *cis*-acting elements present between −538 to −514 and −148 to −124 regions act synergistically or additively in enhancing the *c-jun* transcription, a reporter construct was made in which the Jun-25b spanning −538 to −514 region of *c-jun* was cloned upstream of the plasmid p148*jun*-eGFP. The construct p148*jun*25b-*e*GFP, thus made, consisted of both the above elements. The transient transfection analysis data with this construct shown in Fig. 3 clearly demonstrate that the two elements do not act synergistically and the enhancement observed with p148*jun*25b-*e*GFP (∼125%) was comparable with that observed with the p123*jun*25b-eGFP (∼111%). Thus, enhancement brought about by the −538 to −514 region remained dominant. It is possible that when the two elements are present so close together, the *trans*-acting factors interacting with the −148 to −124 region are not able to bind efficiently as

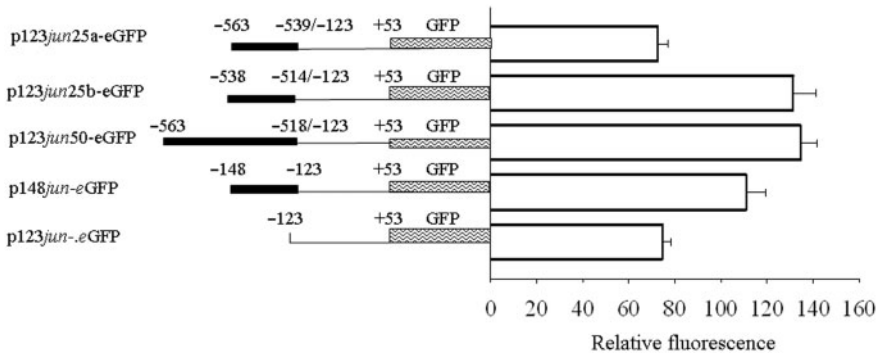

Fig. 2. **Delineation of the positive regulatory element within the −563 to −514 region of *c-jun*.** CHO cells were transfected with the plasmids harbouring −563 to −514, −563 to −539 and −538 to −514 regions of *c-jun* cloned upstream of *c-jun* promoter. Schematic representations of various plasmids used are given on the left. Plasmids p123*jun*-eGFP and p148*jun*-eGFP were used as controls. The data represent the ratio of the observed fluorescence to β-galactosidase activity in the respective samples. Relative fluorescence represents mean ± SD of three independent transfections performed in triplicates for each of the plasmids.

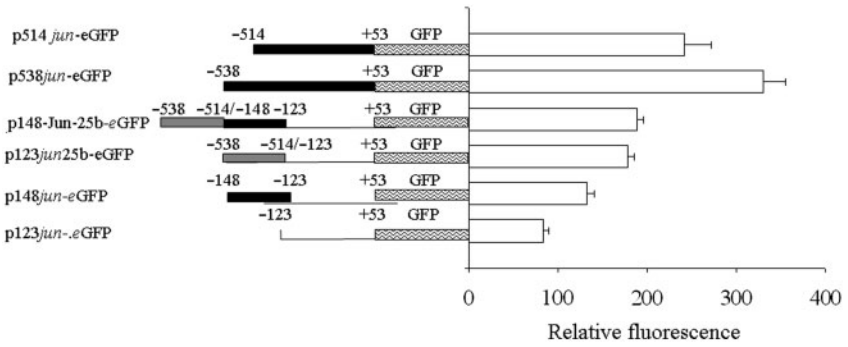

Fig. 3. **Establishment of the role of −538 to −514 region of *c-jun* on the total *c-jun* promoter activity.** Transfection was carried out in CHO cells using plasmids harbouring −538 to +53 (p538*jun*-eGFP) and −514 to +53 (p514*jun*-eGFP) region of *c-jun* to analyse the role of Jun-25_{SA} in overall *c-jun* transcription. To assess the synergistic or additive effect of the two elements spanning −538 to −514 and −148 to −124 region of *c-jun*, transfection was also carried out using chimeric promoter construct, p148*jun*25b-*e*GFP. Plasmids pCMV-GFP, p123*jun*25b-eGFP and p148*jun*-eGFP plasmid were used as positive controls. The data represent the ratio of the observed fluorescence to β-galactosidase activity in the respective samples. Relative fluorescence represents mean ± SD of three independent transfections performed in triplicates with the respective plasmids.

the sequences/DNA are masked by the *trans*-acting factors interacting with the −538 to −514 region. Comparison of binding characteristics of the *trans*-acting factors interacting with the two regions (−538 to −514, this study and −148 to −124 region, earlier studies) clearly indicate that the *trans*-acting factors interacting with the −538 to −514 region bind with their cognate sequence with greater affinity in comparison to that interacting with the −148 to −124 region of *c-jun*.

Sequence comparison of the two regions did not show any similarities. Unlike the positive regulatory element located within −148 to −124 region, no palindromic sequences were observed in the −538 to −514 region (*20*); however, the presence of GGCC at both the ends of the positive regulatory element spanning −538 to −514 region was observed. These data suggest that the transcription regulation of *c-jun* by the element present within −538 to −514 region of *c-jun* is probably mediated by interaction with an entirely different set of transcription factor(s). MAPPER database analysis of the DNA sequence of −538 to −514 region revealed that this region could potentially be recognized by the cAMP response element-binding protein, CREB, a bZip family of transcription factor (Swiss Prot Acc. No: P16220), RAS-responsive element-binding protein 1 and RREB-1, a zinc-finger motif enhancer-binding protein of *Homosapiens* (Swiss Prot Acc. No: Q92766) (*38*). It is to be noted that both these factors are known to be positive-regulatory *trans*-acting factors of the target genes. Thus, the involvement of multiple regulatory elements further upstream of the promoter defines the complexity associated with the tight regulation underlying *c-jun* transcription. The present findings demonstrate that *c-jun* can also be regulated by *cis*-acting elements very distinct from the well known AP-1, SP-1 or NF-jun or RLjunRP.

Binding Characteristics of trans-acting Factor(s) Specifically Interacting with the −538 to −514 Region of c-jun—Since the 25 bp region present between −538 and −514 significantly stimulated transcription from *c-jun* promoter, it was important to establish if this region is recognized by sequence-specific *trans*-acting factor(s), present in rat liver nuclear extract (RNE-d) if any. In order to establish this, EMSA was carried out using region-specific radiolabelled oligonucleotide, referred to as Jun-25_{SA} henceforth. Figure 4A shows that the complex formation between the factor(s) present in the RNE-d and the Jun-25_{SA} was achieved over a wide range of nuclear protein (25–200 μg nuclear protein, lanes 1–4, respectively). The fact that the *trans*-acting factors involved in transcription regulation are generally highly specific for its recognition sequence prompted us to determine the specificity of interaction between the Jun-25_{SA} and the *trans*-acting factors present in the RNE-d. It was observed that pre-incubation of the nuclear extract with increasing concentrations of the cold oligonucleotide, Jun-25_{SA}, resulted in a corresponding decrease in the complex formation, whereas pre-incubation of nuclear extract with much higher concentrations (up to 2,000-fold) of non-specific fragmented SS-DNA did not affect the complex formation (Fig. 4B). Presence of a high intensity complex even in the absence of SS-DNA revealed significant affinity of the *trans*-acting factor(s) to its cognate recognition sequence which enables it to compete with the non-specific DNA-binding proteins present in the extract (Fig. 4B, lane 1). The specificity of the interaction between Jun-25_{SA} and *trans*-acting factor(s) was also adjudged by the addition of a non-specific protein (BSA) and an unrelated oligonucleotide (30 bp) prior to the addition of labelled specific oligonucleotide. Absence of any complex formation in both the cases obviated the possibility of interaction of any non-specific proteins with the Jun-25_{SA} (data not shown). No complex formation was observed in the EMSA performed with an oligonucleotide corresponding to the −563 to −539 region of *c-jun* (data not shown).

As the modulation of transcription by *trans*-acting factors is generally brought about by interaction with the *cis*-acting elements, the absence of any complex between the *trans*-acting factors present in the nuclear extract and this oligonucleotide, thus, support our earlier transfection data, where this element when cloned upstream of the *c-jun* basal promoter failed to exert

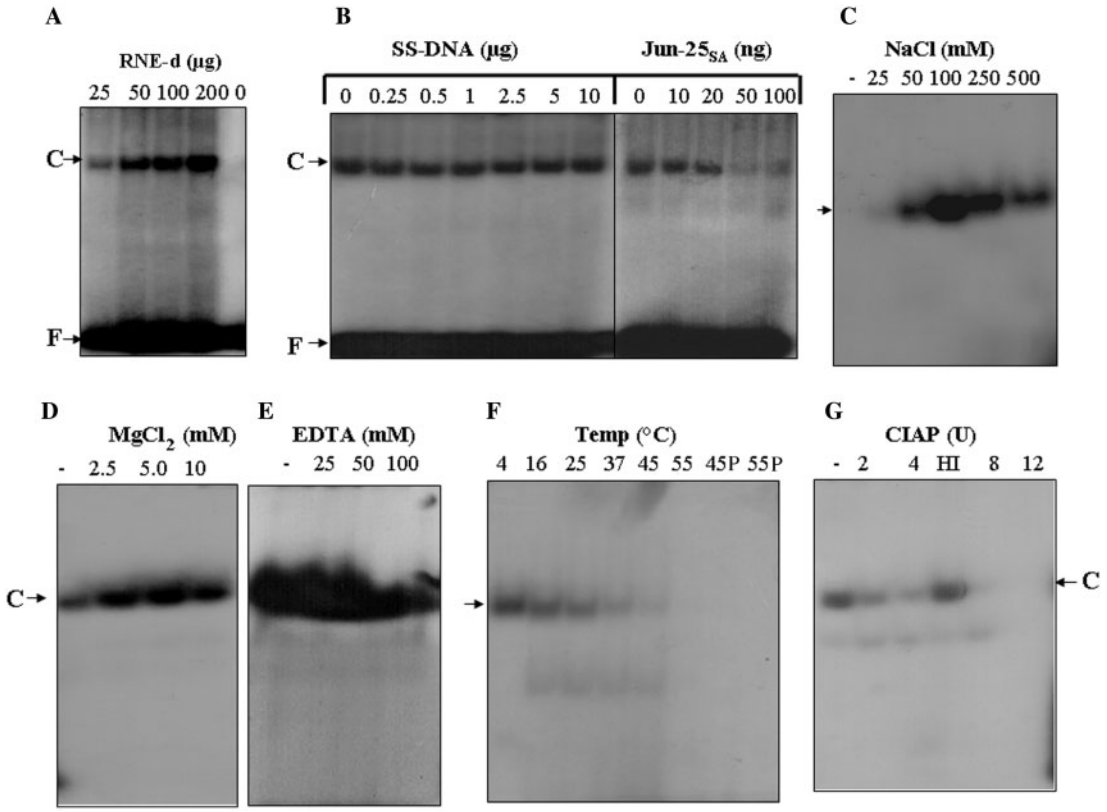

Fig. 4. **(A) Determination of optimum concentration of nuclear extract for complex formation.** EMSA was performed with 2 ng of radiolabelled Jun-25_{SA} and different concentrations (given on top) of rat liver nuclear extract fraction RNE-d. (B) Specificity of complex formation between *trans*-acting factor(s) present in RNE-d and Jun-25_{SA}. EMSA was carried out using 100 µg RNE-d pre-incubated with unlabelled Jun-25_{SA} or fragmented SS-DNA prior to the addition of radiolabelled Jun-25_{SA} in the reaction. (C) Determination of optimum monovalent ion concentration. EMSA was performed using 2 ng Jun-25_{SA} and 100 µg RNE-d in the presence of varying concentrations of NaCl. (D and E) Determination of requirement of divalent cations for complex formation. EMSA using 2 ng Jun-25_{SA} and 100 µg RNE-d was performed in the presence of different concentrations of $MgCl_2$ (2.5–10 mM) or EDTA (25–100 mM). (F) Thermostability of complex formation. EMSA using Jun-25_{SA} (2 ng) and RNE-d (100 µg) was set up over a wide range of temperature (0–55°C. '45P' and '55P' lanes represent EMSA carried out with RNE-d, pre-incubated at 45°C and 55°C for 30 min, respectively. (G) Phosphorylation of *trans*-acting factors is imperative for its interaction with DNA. One-hundred micrograms of RNE-d was treated with different concentrations of CIAP (shown at the top) prior to its use in EMSA. 'HI' indicates EMSA reaction performed with RNE-d treated with 12 U of heat inactivated CIAP. 'C' in all the panels indicate the DNA–protein complex, whereas 'F' indicates unbound radiolabelled Jun-25_{SA}.

any effect on GFP expression. On the other hand, a highly specific complex between the *trans*-acting factors present in the RNE-d and the oligonucleotide spanning the −538 to −514 region supports the observed stimulatory effect of this region in transcription from *c-jun* promoter in transfection analysis.

DNA-binding activities of the known transcription factors are markedly modulated by the addition of monovalent and divalent cations at physiological concentrations. It has been reported that the interaction of AP-1 to its recognition site occurs at an optimum concentration of KCl and $MgCl_2$ and in a temperature-dependent manner in brain nuclear extracts (*39*). Binding of CREB-B-Zip domain to CRE DNA sequence in the presence of either $MgCl_2$ or KCl obliterates binding to non-consensus DNA sequences, but does not appreciably affect the binding of CREB to the CRE-binding site, suggesting the involvement of these cations for sequence-specific DNA binding (*40*). Therefore, role of both the monovalent and divalent cations was evaluated in the interaction of the *trans*-acting factor(s) with the −538 to −514 region. When EMSA reactions were carried out in the presence of different concentrations of NaCl, complex formation was observed over a wide range of NaCl (50–500 mM) with an optimum complex formation in the presence of 100 mM NaCl (Fig. 4C). The factor(s) interacting with this region appears to be different from the one interacting with the positive regulatory element spanning −148 to −124 region of *c-jun*, reported earlier from our laboratory. The RLjunRP, the positive regulatory *trans*-acting factor interacted with the −148 to −124 region in the presence of up to 250 mM NaCl, and no complex formation was observed at 500 mM (*20*), whereas the *trans*-acting factors interacting with the −538 to −514 region were able to form the complex even in the presence of 500 mM NaCl, indicating higher affinity to its recognition sequence. It is evident that the monovalent cations are absolutely essential for complex formation as no complex was formed in the absence of NaCl (Fig. 4C, lane 1). EMSA carried out in the presence of

varying concentrations of $MgCl_2$ and EDTA clearly indicated that the presence of Mg^{++} is not essential for complex formation as a complex could be seen even in the absence of exogenous $MgCl_2$. Addition of $MgCl_2$ at concentrations as low as 2.5 mM not only enhanced the complex formation, but also completely abolished the formation of non-specific complexes. Moll *et al*. have also reported that the presence of Mg^{++} in EMSA reactions prevented non-specific electrostatic interactions between the proteins and DNA, thereby reducing the non-specific complex formation by ~1,000-fold (*40*). No significant increase in the complex formation was observed with further increase in $MgCl_2$ concentrations (Fig. 4D, lanes 1–4). When EMSA was carried out in the presence of EDTA, no effect on the complex formation was observed at 25 mM EDTA. A decrease in the complex formation was observed with increasing concentrations of EDTA, i.e. 50 mM and 100 mM (Fig. 4E). It appears that 25 mM EDTA is not sufficient to chelate the divalent cations, and hence the complex formation remains almost unaffected. However, raising the EDTA concentration to 100 mM possibly resulted in chelating a fraction of the cations, and thereby affected the complex formation slightly. These data suggest that the divalent ions are not absolutely essential for complex formation. However, they may in some way, facilitate the DNA–protein interaction. Similar effect of Mg^{++} and other divalent ions (Zn^{++}) on DNA–protein interaction have been reported earlier (*41*, *42*).

Complex formation at varying temperature conditions suggested that the interaction of Jun-25_{SA} with the specific *trans*-acting factor(s) is highly sensitive to the changes in temperature. The complex was quite stable in temperatures ranging from 4°C to 25°C (Fig. 4F, lanes 1–3). The complex formation reduced drastically at 37°C (lane 4) and was completely abolished at 45°C (lane 5). This is in accordance with the complex forming abilities of other transcription factors that become totally inactivated at 47°C within 15 min (*43*). To establish that whether this sudden decrease was due to inactivation of proteins in the fractionated RNE-d or due to the adverse effect on the interaction of protein and Jun-25_{SA} at higher temperatures, the RNE-d was incubated at 45°C and 55°C for 30 min prior to EMSA at standard conditions (lanes 7 and 8). These data suggest that failure of complex formation at higher temperatures was due to the inactivation of protein factors (Fig. 4F).

Activity of transcription factors is generally regulated by phosphorylation and dephosphorylation. Their switch 'on' or 'off' state mediated by either phosphorylation or dephosphorylation can have variable consequences on the downstream effector pathways (*44*). In CAAT/enhancer-binding protein homologous transcription factor (CHOP), phosphorylation at different site has been reported to have entirely opposite effects on its *trans*-activation potential (*45*). While p38-MAP kinase mediated phosphorylation at serine 79 and serine 81 augmented the transcriptional activity of CHOP, phosphorylation of its amino terminus by casein kinase II inhibited its transcriptional activity (*45*, *46*). Therefore, it was imperative to assess whether the *trans*-acting factor(s) interact with the 25 bp *cis*-regulatory element of *c-jun* either in phosphorylated or dephosphorylated state. A gradual decrease in the intensity of the complex formation upon dephosphorylation of the rat nuclear extract proteins using CIAP, suggests that the *trans*-acting factors bind to their cognate DNA only in the phosphorylated form (Fig. 4G, lanes 2, 3, 5 and 6). Denaturation of CIAP prior to its addition in EMSA reaction did not affect the complex formation (lane 4), thereby confirming that the decrease in the intensity of the complex formation in the presence of native CIAP is indeed due to dephosphorylation of the specific protein factor (s) interacting with Jun-25_{SA}. Our findings are also in line with earlier reports where dephosphorylation completely inhibited the binding activity of RLjunRP with its recognition sequence (*20*).

The trans-acting Factor(s) Interacts with the –538 to –514 Region of c-jun Through Minor Groove Binding—To address whether the *trans*-acting factor(s) interact with DNA in the major groove or minor groove, the influence of site specific drugs on complex formation was analyzed. It was observed that low concentrations of distamycin A (0.1–10 μM), a minor groove binding drug, did not affect the DNA–protein interaction when compared to the control (Fig. 5A, lanes 12–17). However, 25 μM distamycin A completely inhibited the complex formation (lane 18). It is likely that distamycin A inhibits the complex formation either by competing for binding to the minor groove of DNA or by altering the local DNA conformation which weakens the binding affinity of the *trans*-acting factors. In contrast, methyl green, a major groove binding drug, did not alter the complex formation even at concentrations as high as 200 μM, when compared to the control, confirming that the protein–DNA adduct formed is indeed via minor groove interactions (Fig. 5A, lanes 1–10). Other DNA binding proteins such as TATA-box-binding protein, integration host factor (IHF), high mobility group I(Y)[HMG I(Y)] and the HMG-box-containing proteins, SRY and LEF-1 have also been reported to interact with their target sites exclusively through minor groove contacts (*47*, *48*).

The minimal region required for complex formation within the $Jun25_{SA}$, was determined by performing EMSA with oligonucleotides consisting of 10 bp deletion from either the 5' or the 3'-ends in the $Jun25_{SA}$ ($Jun25_{SA}$-1 and $Jun25_{SA}$-2, respectively). The *trans*-acting factors interacting with the Jun-25_{SA} were unable to form complex with the deletion variants of the Jun-25_{SA} (Fig. 5B). The absence of an adduct formation with either of the deleted oligonucleotides clearly reflects that the binding of the specific *trans*-acting factor(s) to the 25 bp region is a result of the nuclear protein association over the full length of the DNA.

Molecular Mass Determination of the trans-acting Factors Interacting with the –538 to –514 Region of c-jun—UV cross-linking analysis of the DNA–protein adducts formed between the Jun-25_{SA} and *trans*-acting factors revealed presence of three complexes of approximate molecular mass of ~80 kDa, ~45 kDa and ~34 kDa, the ~80 kDa adduct being of the maximum intensity (Fig. 6A). The data demonstrated that the two proteins of ~45 kDa and ~34 kDa are able to interact with the Jun-25_{SA} independently, albeit a weaker interaction is reflected for the ~45 kDa protein. The majority of the

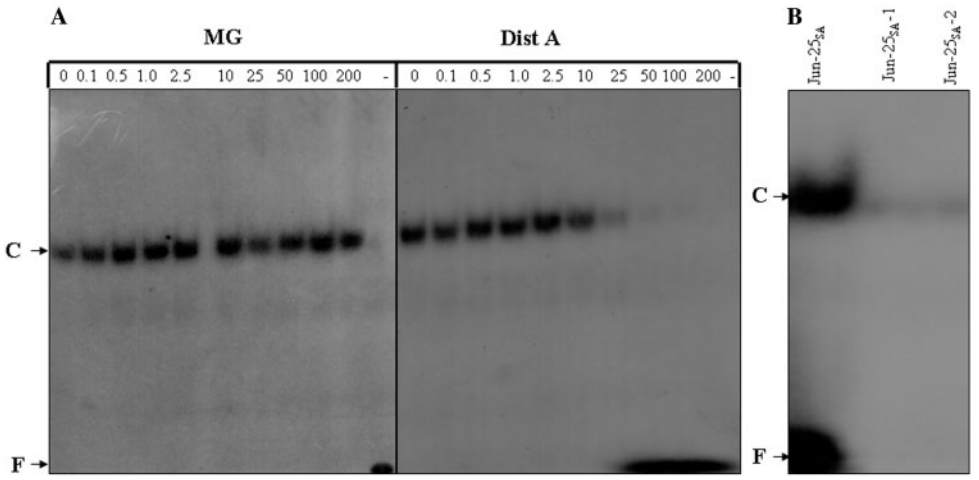

Fig. 5. **Binding site determination of the *trans-acting* factors interacting with the −538 to −514 region of *c-jun*.** (A) Effect of distamycin A, Dist. A and methyl green, MG on complex formation. Standard EMSA reactions were carried out using 2 ng of Jun-25_{SA} and 100 μg RNE-d in the presence of different concentrations (micromolar) of distamycin A or methyl green. The reaction products were separated on 5% non-denaturing PAGE. (B) EMSA with deletion variants of Jun-25_{SA}. Standard EMSA reactions using 2 ng of Jun-25_{SA} and its deletion variants and 100 μg RNE-d. Jun-25_{SA}-1 and Jun-25_{SA}-1 represent oligonucleotides with 10 bp deletions in the Jun-25_{SA} from the 5′- and 3′-ends, respectively.

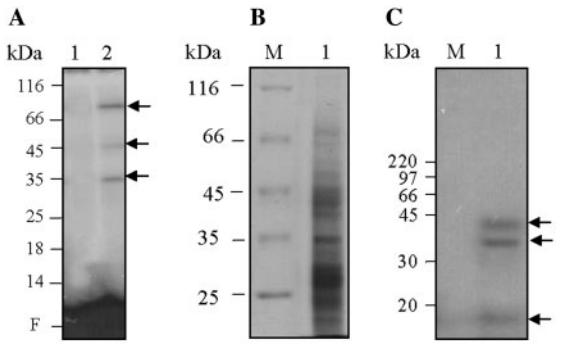

Fig. 6. **Molecular mass determination of *trans*-acting factors interacting with −538 to −514 region of *c-jun*.** (A) UV cross-linking analysis. Standard EMSA was performed using 5 ng radiolabelled Jun-25_{SA} and 200 μg BSA and RNE-d (lanes 1 and 2, respectively). The reaction products were UV-irradiated at 254 nm as described in the MATERIALS AND METHODS section and separated on 12% SDS–PAGE. The arrows points to the UV cross-linked complexes formed between *trans*-acting factor(s) present in RNE-d and Jun-25_{SA} (lane 2). F indicates unbound radiolabelled Jun-25_{SA}. Migration of protein molecular weight marker (kiloDaltons) is shown on left. (B) SDS–PAGE analysis of RNE-d: lane M and lane 1 indicate protein molecular weight markers and RNE-d, respectively. Proteins were detected by staining the gel with Coomassie Brilliant Blue R250. (C) South-western blot analysis of RNE-d. Proteins present in RNE-d (lane 1) resolved on 12% SDS–PAGE were transferred to nitrocellulose membrane and hybridized with radiolabeled Jun-25_{SA}. Arrow points to the hybridized bands. Unstained mid-range protein molecular weight marker was used in (A and B), whereas broad range pre-stained protein molecular weight marker was used in (C). Migration of the molecular weight marker is shown on the right (kDa).

cross-linked species at ∼80 kDa could possibly be due to a hetero/homodimer of the two proteins of ∼45 kDa and ∼34 kDa, or due to an interaction of the Jun-25_{SA} with yet another protein of ∼80 kDa.

In order to determine if the Jun-25_{SA} interacts directly with the proteins of ∼45 kDa and ∼34 kDa or also with a protein of ∼80 kDa, South-western blot analysis was carried out using RNE-d and Jun-25_{SA} as a probe. SDS–PAGE analysis of fraction RNE-d clearly shows the presence of large number of nuclear proteins (Fig. 6B). However, only two bands at ∼45 kDa and ∼34 kDa were detected in South-western blot analysis of the same using the Jun-25_{SA} a probe (Fig. 6C). In addition, small molecular mass complex at a position lower than ∼20 kDa was detected which could possibly be due to the interaction of Jun-25_{SA} with the histone/non-histone chromatin proteins abundant in nuclear extracts, which have the general ability to bind to the DNA. Further, the absence of any DNA adduct at the positions lower than ∼20 kDa in the UV cross-linking analysis suggest that these low molecular mass proteins detected in South-western blot are indeed due to non-specific interaction. Absence of any∼80 kDa band in the South-western blot ruled out the possibility of interaction of Jun-25_{SA} with a protein of molecular mass of ∼80 kDa. Thus, from the South-western blot analysis and UV cross-linking data, it can be inferred that the ∼80 kDa adduct detected in UV cross-linking is due to the formation of either hetero or a homodimer of the two proteins of ∼45 and/or ∼34 kDa. Many transcription factors exert their transcriptional activity either through homodimerization or heterodimerization with a protein partner. AP-1, NFκB, NF-jun and v-erbA are only to name a few of the transcription factors that activate transcription of the target genes through binding of a dimer to their recognition sequence (*6*, *49–51*). The activity of a transcription factor can altogether be different depending on the dimerization partner.

DNA-affinity Purification of the trans-acting Factors Interacting with the −538 to −514 Region of c-jun— Purification of the *trans*-acting factor(s) interacting with Jun-25_{SA} from the fractionated RNE-d was accomplished

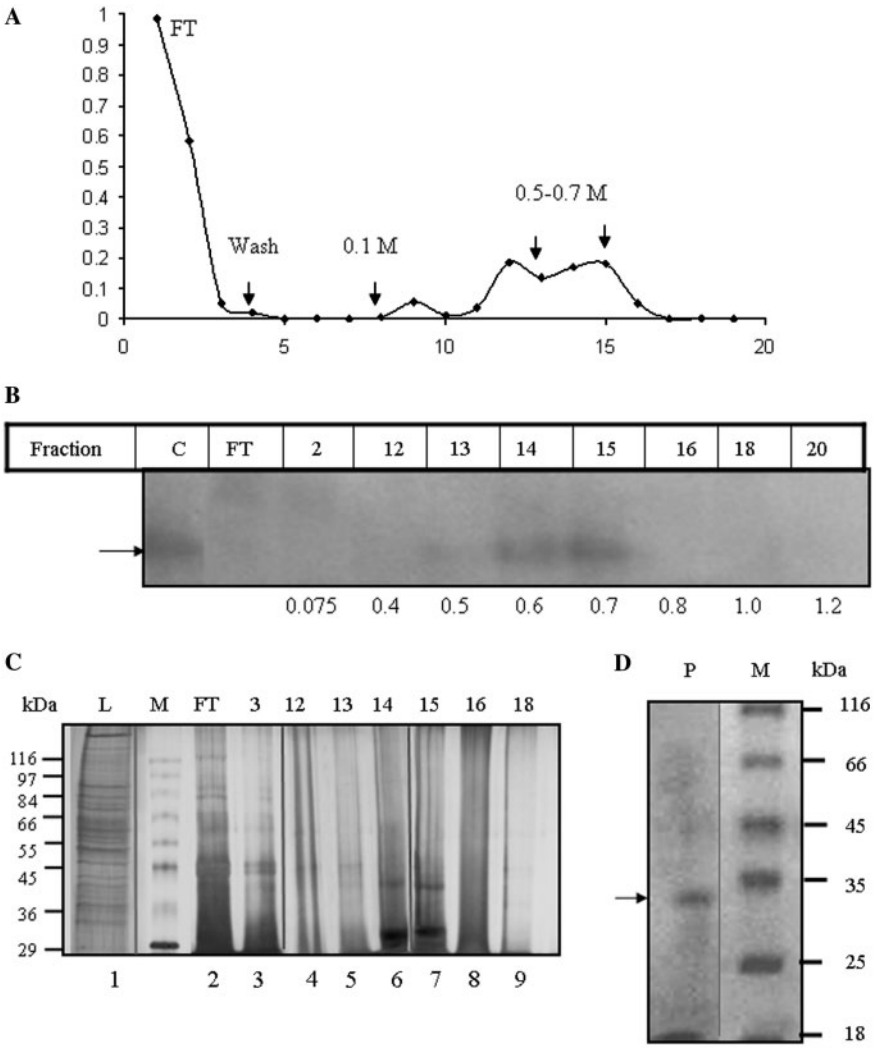

Fig. 7. **Affinity purification of the *trans*-acting factors interacting with −538 to −514 region of *c-jun*.** (A) Spectroscopic analysis of protein fractions. Fraction RNE-d was subjected to sequence specific DNA-affinity chromatography. Fractions 4–7 (1 ml each) represent wash fractions (four washes with buffer containing 75 mM NaCl). The bound proteins were batch eluted with buffer containing 0.1–1.2 M NaCl. All the fractions were analysed spectrophotometrically. A_{280} of different fractions was measured and plotted. (B) Analysis of complex forming ability of different fractions obtained from DNA-affinity chromatography. Standard EMSA reactions were performed using different fractions and radiolabeled Jun-25_{SA}. 'C' represents EMSA performed with RNE-d used for affinity chromatography. FT indicates flow-through fraction. The numbers on the top represent the fraction number and at the bottom represent salt concentration in the respective fraction. (C) SDS–PAGE analysis of affinity purified fractions. Different fractions (fraction number given on top of the gel) obtained from DNA-affinity chromatography were resolved on 12% SDS–PAGE and subjected to silver staining. 'L' indicates load fraction RNE-d and 'M' indicates protein molecular weight marker. (D). Coomassie brilliant blue staining of affinity purified proteins on SDS–PAGE. The fractions 14 and 15 eluted with 0.6 M and 0.7 M NaCl, respectively and exhibited the complex forming ability, were pooled and resolved on 12% SDS–PAGE and analysed by coomassie brilliant blue staining. 'P' indicates the pooled purified fractions 14 and 15. 'M' indicates the mid range protein molecular weight marker. The arrow points to the predominant ~34 kDa protein that interacted with Jun-25_{SA}. 'kDa' indicates the migration of protein marker.

by DNA-affinity chromatography. The proteins bound to 25 bp oligonucleotide were eluted with different concentrations of NaCl (Fig. 7A). Major peak fractions were observed between 0.4 M and 0.7 M NaCl. EMSA using different fractions revealed that the proteins eluting at NaCl concentrations lower than 0.5 M did not show any complex formation with radiolabelled Jun-25_{SA} (Fig. 7B). Presence of specific complexes in fractions eluting with 0.6–0.7 M NaCl confirmed the presence of *trans*-acting factors specifically interacting with the Jun-25_{SA} (Fig. 7B, lanes 6 and 7). The elution of specific DNA-binding proteins at concentrations higher than 500 mM NaCl was in line with the EMSA carried out at varying NaCl concentrations (refer to Fig. 4C). SDS–PAGE analysis of the fractions exhibiting the ability to form complex with Jun-25_{SA} revealed the presence of a predominant band at ~34 kDa in these fractions. A band at ≈ 45 kDa could also be seen in these lanes (Fig. 7C, lanes 6 and 7). It is to be noted that a faint band at the ~45 kDa position could also be seen in the fraction

eluting at 0.4 M NaCl (Fig. 7C, lane 5), whereas majority of the ~34 kDa protein was eluted with 0.6 M and 0.7 M NaCl. An additional band at a position higher than ~45 kDa in the fraction eluted with 0.4 M NaCl appears to be non-specific, as its absence in 0.6 M NaCl and 0.7 M NaCl fractions did not affect the complex formation. Coomassie staining of the proteins present in fraction showing specific complex formation (eluted with 0.6 and 0.7 M NaCl) on an SDS–PAGE, electrophoresed for a shorter period, confirmed the predominance of the ~34 kDa protein in the fractions (Fig. 7D, lane 'P'). These data indicate that the ~34 kDa protein is more abundant in the nuclear extract in comparison to the ~45 kDa protein. Absence of ~80 kDa protein in the affinity purified fractions further confirmed that the ~80 kDa DNA–protein adduct detected in the UV cross-linked sample is not due to the interaction of Jun-25_{SA} with a ~80 kDa protein and rather due to hetero/homodimerization of the ~45 kDa and ~34 kDa *trans*-acting factors. It appears that the ~34 kDa protein is not only abundant in the nuclear extract but also interacts more strongly than the ~45 kDa protein, as the ~45 kDa band could elute at much lower NaCl concentration during affinity purification. Also, the DNA–protein adduct at ~45 kDa position is much weaker than that at the ~34 kDa position in UV cross-linking analysis. It is possible that it is the ~34 kDa protein that interacts with the Jun-25_{SA} directly, and the ~45 kDa protein interacts both with the DNA and also with the ~34 kDa protein complexed with the DNA, thus giving rise to a ~80 kDa DNA–protein adduct. Identification of the two proteins and their production through recombinant route will help ascertain if this is the case or that the ~80 kDa adduct is due to the binding of ~34 kDa dimer to Jun-25_{SA}.

It has also been reported earlier that different *trans*-acting factors interact with *cis*-acting element under different physiological conditions and may vary among different cell types (*20*, *49*). Therefore, EMSA using nuclear extracts prepared from normal and regenerating liver was performed to determine if the −538 to −514 region involved in the positive regulation of *c-jun* is differentially recognized by factors present in quiescent and proliferating rat liver. However, no difference in the complexes was noted between the two (data not shown), suggesting that unlike the −148 to −124 region (*20*), the −538 to −514 region of *c-jun* is not involved in enhanced *c-jun* expression in proliferating cells. Being a component of the transcription factor AP-1, c-Jun plays a crucial role in cell proliferation, differentiation and cell death; it is likely that the *trans*-acting factor(s) interacting with the −538 to −514 region could be involved in regulation of *c-jun* in a cellular condition(s) other than proliferation.

Earlier studies have analysed the upstream regions of *c-jun* only up to 290 bp from the transcription initiation site for their recognition by the *trans*-acting factors. The present study has thus established the −538 to −514 region of *c-jun* as a positive regulatory *cis*-acting element involved in the transcriptional regulation of *c-jun*, adding to the existing knowledge of the highly complex molecular switches, indispensable for *c-jun* regulation under different physiological conditions. Large-scale purification of the *trans*-acting factor(s) interacting with the −538 to −514 region is in progress to identify the protein. Production of the same through recombinant route will enable us to further explore its structural-functional aspects.

ACKNOWLEDGEMENTS

Council of Scientific and Industrial Research (CSIR), India, is duly acknowledged for the Senior Research Fellowship to S.A. and Rajkumar. UGC is acknowledged for the research fellowship to P.G. Technical Assistance provided by Mr Amresh Kumar Singh and Mr Umesh Roy is sincerely appreciated. The animal experiments carried out in this work had the approval of Institutional Animal Ethics Committee, JNU (IAEC-JNU Project Code No. 01/2005).

FUNDING

Department of Biotechnology (DBT) and University Grants Commission (UGC), India.

CONFLICT OF INTEREST

None declared.

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