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Sp1 is essential and its position is important for p120 gene transcription: a 35 bp juxtaposed positive regulatory element enhances transcription 2.5 fold

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ABSTRACT

Human proliferating cell nucleolar antigen p120 is expressed in tumor cells in the early G1 phase of the cell cycle. Deletion analyses of the essential cis-acting region −537/−278 showed that a 58 bp sequence from −457 to −400 is an important cis-acting element. An Sp1 transcription factor binds to the sequence AGAGGCGGGG (−425 to −416) within the −458/−400 cis-acting region. Deletion of the Sp1 binding sequence eliminated transcription. Substitution of the Sp1 box(−437/−406), containing the Sp1 recognition site, for the entire cis-acting region (−537/−278) restored transcription only at a very low level (18%). Deletion of the −537/−278 cis-acting region followed by substitutions showed that the Sp1 box (−437/−406) stimulated transcription 2.4 fold, when juxtaposed and downstream of a 35 bp (−472 GGGCGAGCGTAAGTTCCGGGTGCGGCGGCCGACTA −438) positive regulatory cis-element (PRE) over that by substitution of the Sp1 box alone. When the −406/−278 sequence was downstream of the PRE-Sp1 box, transcription was stimulated 4.4 fold over that produced by substitution of the Sp1 box alone. These results suggest that Sp1 is essential and its proper position in the 5′ flanking sequence, juxtaposed and down stream of a 35 bp positive regulatory sequence, is required for efficient transcription.

INTRODUCTION

P120 protein is a human proliferating cell nucleolar antigen which appears early in the G1 phase of the cell cycle (1). Protein p120 is one of the most interesting of the proliferating cell nucleolar antigens(1−6). Ochs *et al.* (7) found that the p120 antigen is localized to the beaded microfibrillar network of the nucleolus and suggested that the p120 antigen might be a component of the nucleolar matrix of the highly pleomorphic and functionally hyperactive nucleoli of tumor cells(8). The p120 antigen is detected in most human malignant tumors and tumor cell lines including cancer of the liver, lung, breast and lymphoid tissues, but not in most normal resting tissues (1). Human tumor cell lines contain 15−60 times more p120 messenger RNA than does human term placenta (9). In mitogen activated lymphocytes, p120 is detected in the early G1 phase of the cell cycle (10). Freeman *et al.* (11) analyzed p120 in a large number of breast tumor specimens and reported that p120 antigen is an important marker for breast tumor progression. Perlakey *et al.* (unpublished data) in our laboratory showed increased growth of NIH/3T3 cells by transfection with human p120 expression vector and inhibition by a p120 antisense construct.

Fonagy *et al.* (12) determined the p120 cDNA sequence, and Larson *et al.* (13) determined the genomic sequence. The p120 protein contains, consecutively, four major domains: a basic domain, an acidic domain, a hydrophobic and methionine-rich domain and a domain rich in cysteine and proline residues (12). Protein p120 is phosphorylated at Thr−185 and /or Ser −181 (14). Haidar *et al.* (15) recently reported that two cis-acting regions are important for the efficient transcription of the p120 gene. The first of these regions, −537/−278, is an absolute requirement for initiation of transcription (15). The other region, −1426/−1223, stimulates transcriptional activity two fold (15). Within this region a protein factor binds to a purine rich sequence −1353/−1332 (AAAG*AGGAGGAGG*TAAGTGGCA) (16). The present report shows that an Sp1 transcription factor is essential and its proper position with respect to the transcription initiation site is important for optimum transcription. A juxtaposed 35 bp (GGGCGAGCGTAAGTTCCGGGTGCGGCG-GCCGACTA) positive regulatory cis-acting element stimulated transcription 2.4 fold.

MATERIALS AND METHODS

Plasmids: The p ▲CAT, p−2532/+98 CAT, p TK CAT, p −537/−278 TK CAT constructs were described earlier (15). The 5′ deletions of the p−537/−278 TK CAT were obtained by Exo III deletions using the Erase a Base System of Promega. The plasmid p sSp1 was constructed by substituting a 32 bp oligo containing the Sp1 binding sequence (−437 GGCGCAACAGG-A*AGAGGCGGGG*CCGCGAGCGT −406; the underlined sequence is the Sp1 binding site) for the −537/−278 cis-acting region of the p−2532/+98 CAT construct (in this construct the p120 coding sequence was in frame with the CAT coding sequence, confirmed by sequencing). All the constructs described below were derived from p−2532/+98 CAT construct and are in frame with the CAT coding sequence. The p *sSp1 was

constructed by substituting the same 32 bp oligo except that it had five mutations within the Sp1 binding sequence (GGCGC-AACAGGAAGA*TA*CG*AGCA*CGCGAGCGT, the mutated nucleotides are underlined). p s35Sp1 was made by inserting a 35 bp wild type sequence (−472 GGGCGAGCGTAAGTT-CCGGGTGCGGCGGCCGACTA −438) upstream of Sp1 in the Sst II site of the p sSp1. p ▲Sp1 was made by deletion of the Sp1 binding sequence, (AGGA*AGAGGCGGGG*CCGC), from the −2532/+98 CAT construct using the Amersham oligonucleotide directed *in vitro* mutagenesis system. The numbering is with reference to ATG as used in the previous paper due to multiple transcriptional initiation sites (15).

Cells and transient transfections

Hela cells were cultured in Dulbecco's modified Eagle medium (glucose 4.5 g/L) and supplemented with 10% newborn calf serum. All ingredients for cell culture were obtained from J.R.H.Biosciences. Hela cells were transfected by the calcium phosphate precipitation method (15, 17). Equimolar (equivalent to 10 ug p ▲CAT) amounts of all constructs were used in each transfection along with 1ug of phMThGH (human growth hormone under the control of metallothionein IIA promoter) as an internal control. pUC-19 vector was added to keep the total DNA content the same in each transfection. CAT assays were done using 20 ug of protein and incubated at 37°C for 6 hours (15,18). Transfection efficiencies in different transfections were corrected by estimating growth hormone secreted into the media using the Hybritech Tandem-RHGH immunoradiometric assay system.

Preparation of Nuclear extract

Hela cell nuclear extract was prepared according to Dignam *et al.* (19) with some modifications. Nuclei were extracted in 2.5 pelleted volumes of extraction buffer (400mM KCl-10mM HEPES-KOH, pH 7.9−1.5 mM $MgCl_2$−0.1mM EGTA−0.5mM DTT, 5% glycerol and 0.5mM PMSF) by slowly stirring for 30 minutes at 4°C followed by centrifugation at 100,000×g. The supernatant was then dialyzed for 4 hours against 50 volumes of 20 mM HEPES-KOH, pH 7.9, 75 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol and 0.5 mM PMSF. The extract was cleared by centrifugation at 25,000×g for 15 minutes.

Gel retardation assays and DNase 1 footprinting

Gel retardation assays were done as described earlier (20) in 25 ul of binding mixture which consisted of ^{32}P labelled DNA fragment (10,000 cpm), 5ug of poly d(I-C), 1 ug of sonicated salmon testis DNA and 5 ug nuclear extract in 10 mM Tris-HCl, pH 7.1−0.1mM EDTA− 10mM 2-mercaptoethanol−0.1% Triton X 100−5% glycerol −80 mM NaCl-3 mM MgCl2. This mixture was incubated on ice for 30 min. and then loaded on a 4% polyacrylamide gel (acrylamide : bis, 30:1) in 0.5×TBE buffer. For competition experiments various amounts of either unlabelled probes or duplex oligos were added at the beginning of the binding reaction. Duplex oligos were made by heating the complimentary strands of oligos in 10mM Tris-pH 8.0−10mM MgCl2 to 70°C for 5 minutes and then slowly cooling to room temperature. DNase I footprinting analyses were done as described (20). Binding reactions (50 ul) were set up using the 5′ end labelled DNA fragment and HeLa nuclear extract as described for the gel retardation assay. After incubation on ice for 30 minutes, the sample was digested with 4ul of 0.25 U/ul DNase I for 2 minutes at 20°C. The reaction was stopped by addition of 4 ul of 50 mM EDTA, then electrophoresed as above, and the gel was electroblotted to a NA45 DEAE membrane (Schleicher & Schuell). Free (F) DNA and bound DNA (B) were eluted from the membrane, precipitated and loaded on a sequencing gel.

RESULTS

Identification of the precise sequences of the −537/−278 region necessary for p120 gene transcription.

To analyze the −537/−278 cis-acting region more precisely, the −537/−278 TK CAT construct was deleted from the 5′ end with Exo III. The deleted constructs were then transfected into HeLa cells and the relative transcriptions were measured by the CAT enzyme activities. Deletion from −537 to −457 had no effect on stimulation of the TK promoter (see the relative transcription of the −537/−278, −494/−278, −478/−278 and −457/−278 TK CAT constructs in Fig. 1). Further deletions to −445 and −429 resulted in a gradual decrease in the stimulation of the TK promoter. The −400/−278 TK CAT construct showed no stimulation of transcription. These deletion data defined the important cis-acting sequence as a 58 bp sequence, −457/−400.

Trans-acting factor binding to the −537/−278 region of the p120 promoter

Fig. 2 shows the DNase I footprinting analysis of the fragment −537/−278 using HeLa nuclear extract. Comparison of lane B (Bound DNA) with lane F (Free DNA), shows the sequence −428 /−407 (GGAAGA*GG*CGGGGCCG) is protected from

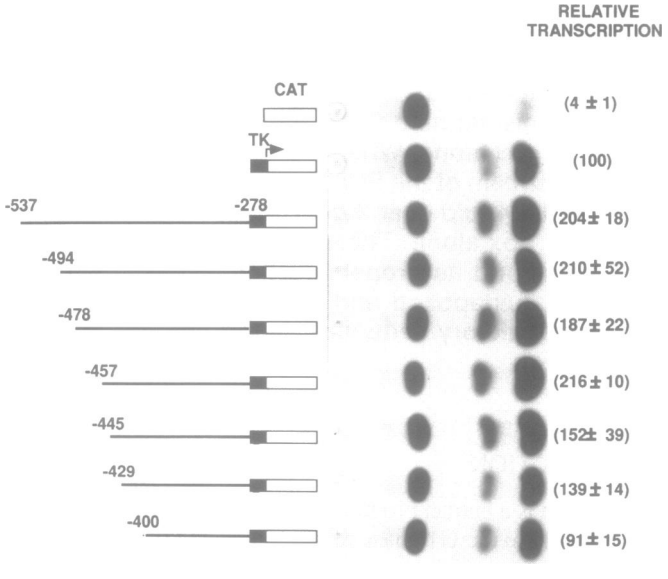

Fig. 1. Deletion analysis of the cis-acting region −537/−278. 5′deletion mutants of the −537/−278 TK CAT constructs were transfected into Hela cells by the calcium phosphate precipitation method (17). HeLa cells were plated at 50−60% confluency in a 100 mM petridish 4−6 hours before transfection. Equimolar (equivalent to 10 ug ▲CAT) amounts of all constructs were used in each transfection along with 1 ug of phMThGH(human growth hormone expression vector) as an internal control.The CAT assays were done using 20 ug of cell extract and incubated at 37°C for 6 hours. The relative transcription efficiencies obtained from CAT assays are shown in parentheses (mean ± SD of 3−4 independent experiments).

DNase I digestion. The nucleotide G at position −422 is strongly protected from DNase I digestion. The sequence of the protected region is similar to GC box sequences of Sp1 recognition sites.

Since the factor binding to the −537/−278 DNA fragment appeared to be an Sp1 transcription factor, competition studies were done with oligos having altered sequences within the GC box. Mutant oligos, within which the GC box sequence was altered, were unable to compete for binding of the protein. Mutation of the single nucleotide G(−422) to T prevented competition (data not shown). The oligo −437/−406 was a better competitor than −429/−412 when competition was done with a 50 ng oligo duplex (data not shown).

Oligos were made to identify the sequences on either side of the G at position −422 which play a role in protein-DNA interaction. Competition studies (Fig 3) showed that three nucleotides (AGA) to the left, and six nucleotides (GCGGGG) to the right of nucleotide G at position −422 are required for this trans-acting factor binding. Hence, the factor binding site is contained within a 10 bp sequence, AGAGGCGGGG.

Deletion of 18 bp sequence, AGGAAGAGGCGGGGCCGC (−429/−412), from the −537/−278 fragment abolished protein factor factor binding (data not shown) in gel retardation assays. This result confirms that the factor is binding to the deleted sequence.

Fig. 4 shows oligo-competition in a gel retardation assay using the −537/−278 region and known Sp1 sequences (250, 500, and 750 ng oligo duplexes were used for competition). Lane 1 shows no competition. Lanes 2−4 show competition with a 16 bp GC rich sequence (−428 GGAAGAGGCGGGGCCG −413) of the p120 promoter. Lanes 5−7 show competition with a known weak Sp1 (GCAGTCGGGGCGGCGCGGTCC) of herpes virus thymidine kinase promoter (21). Lanes 8−10 show competition with a known medium affinity Sp1 binding sequence (GACAC-AAACCCCGCCCAGCGTCT) of the herpes virus TK promoter (21). Lanes 11--13 show competition with an Sp1 sequence (CGCGCGGAGTTACGAGCTCG) of the mouse metallothionine promotor (22,23). The low affinity Sp1 sequence did not compete with the labeled probe but the 18 bp GC box sequence of the

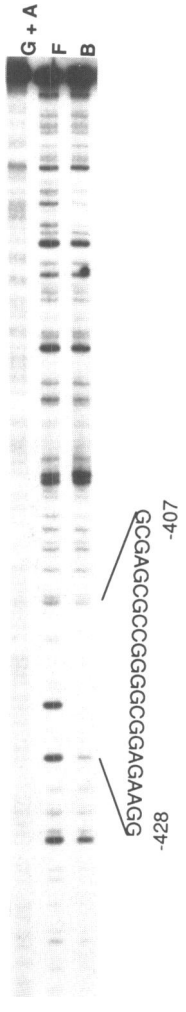

Fig. 2. DNase I footprinting analysis on the −537/−278 fragment. The 5′ end labelled fragment −537/−278 (10 ng), 5 ug of Hela cell nuclear extract, 5 ug of poly d(I-C), and 1 ug of sonicated salmon sperm DNA were incubated on ice for 30 minutes and then digested with DNase I and loaded onto a 4% acrylamide gel as described in the Methods. Following electrophoresis, bound (B) and free (F) DNA fragments were electroblotted onto a DEAE membrane. The DNA was eluted from the membrane, precipitated with ethanol, disolved in 90% formamide containing buffer, and loaded on a sequencing gel. G+A reactions (Maxam Gilbert) of the 5′ end labelled −537/−278 fragment were loaded alongside.

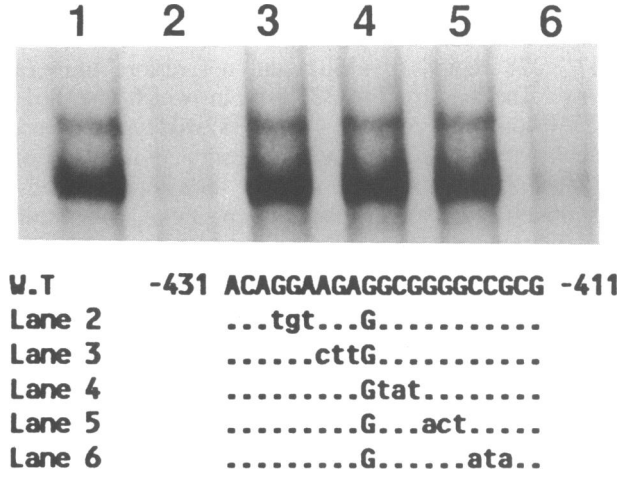

Fig.3. Competition in gel retardation assays with mutant oligos surrounding the nucleotide G at position −422. The ^{32}P labelled −537/−278 DNA fragment was incubated with 5 ug nuclear extract, 5 ug of poly d(I-C), and 1 ug of sonicated salmon sperm DNA in absence and presence of 250 ng of different mutant oligo duplexes as shown in the bottom of this Figure. Oligo duplexes were obtained by annealing the complementary strands as described in the Methods. Capital letters represent the wild type sequence and the small letters represent mutated bases.

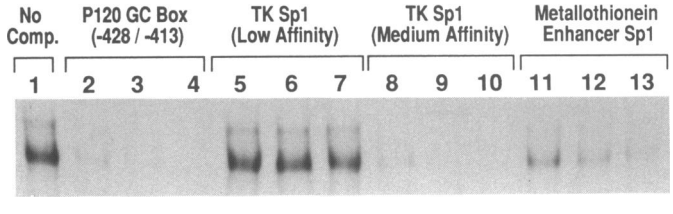

Fig. 4. Competition of Gel retarded bands with known Sp1 sequences. Gel retardation assays were done using end labelled −537/−278 fragment and HeLa nuclear extract in presence or absence of competitor oligos as described in the legends to Fig 3. 250 ng, 500 ng, and 750 ng of different known Sp1 duplex oligos were used in each competition.

p120 promotor, the medium affinity Sp1 of TK promotor, and the Sp1 sequence of the mouse metallothionine promotor competed well. Accordingly, the 10 bp Sp1 binding sequence (AGAGGCGGGG) of the p120 gene is a medium to high affinity Sp1 binding site. The relative affinities of different Sp1 binding sequence of different genes are summarized in reference (24).

Sp1 plays an essential role in the initiation of p120 gene transcription

Fig. 5 shows a diagramatic representation of different deletion and substitution mutations within the −537/−278 region of the −2532/+98 CAT construct and the transcription efficiencies of these constructs. Deletion of the Sp1 recognition site from the −2532/+98 CAT construct (p Sp1) abolished p120 gene transcription, which indicates that this sequence is essential for p120 gene transcription. Substitution of a 32 bp sequence −437 GGCGCAACAGGA*AGAGGCGGGG*CCGCGAGCGT −406 (Sp1 binding sequence is underlined) for the −537/−278 cis-acting region (p sSp1) resulted in only 18% of wild type transcription activity. Thus, the Sp1 box (−437/−406) alone is not able to function efficiently, and requires either one or both of the flanking sequences, −537/−437 and −406/−278, for optimum transcription. Substitution of a mutated Sp1 recognition sequence, GGCGCAACAGGAAGAtaCGaGcaCGtGAGCGT (small letters are the mutated nucleotides) for the same −537/−278 region (p s*Sp1) did not restore transcription activity. The construct p s35Sp1, in which the Sp1 box (−437/−406) and a 35 bp (−472/−438) wild type sequence just upstream of the Sp1 box were substituted in place of the −537/−278 cis-acting region, had 43% of the wild type transcription activity. This construct, p s35Sp1, is 2.4 times as active in transcription as the p sSp1 construct. This 35 bp sequence is a positive regulatory element (PRE). When this PRE sequence was placed in a reverse orientation upstream of the Sp1 box in the p sSp1 construct, transcription was stimulated about 3.0 fold over that caused by p sSp1 (data not shown). The 35 bp PRE sequence did not compete with the −537/−278 fragment for binding with the Sp1 factor (data not shown). Substitution of the −472/−278 sequence for the −537/−278 region (p s-472/−278) showed 77% of the wild type transcription activity indicating that the Sp1 box and both surrounding regions −472/−438 (PRE) and −406/−278, are important for transcription.

Substitution of a foreign DNA sequence of the same length as wild type −406/−278 into the p sSp1 (p sSp1f) enhanced the transcription about 1.5 fold (compare relative transcription of p sSp1 and p sSp1f). The wild type sequence −406/−278 stimulated transcription of p sSp1 construct 1.8 fold (compare p s −472 /−278 and p 35sSp1). Hence the −406/−278 sequence can be replaced with any random sequence of the same length without appreciable change in p120 gene transcription, so the −406/−278 sequence seems to be acting as a 'spacer'.

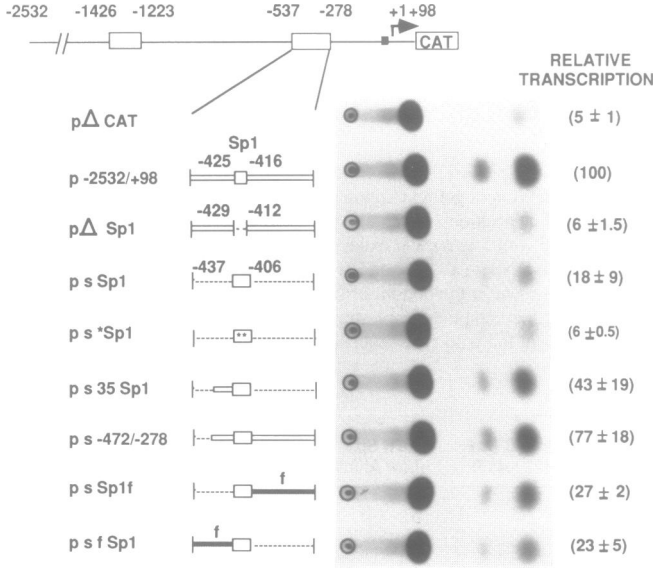

Fig. 5. Role of Sp1 binding sequence in p120 gene transcription. Different deletion and substitution mutations were made within the −537/−278 region of the p −2532/+98 construct as shown diagrammatically and described in the Methods. These constructs were transfected into Hela cells and assayed for the CAT enzyme activity as described in the legends to Fig 1 and Methods. Dashed lines represent deleted sequences. Asterisks represent mutated nucleotides. Data represents mean ± SD of 3−4 independent experiments.

DISCUSSION

This report presents data showing that: a) Sp1 is absolutely required for the initiation of p120 gene transcription, b) a 35 bp sequence (−472-GGGCGAGCGTAAGTTCCGGGTGCGGCG-GCCGACTA −438) adjacent to, and upstream of, the Sp1 box sequence (−437/−406) is a positive regulatory cis-acting element (PRE) of p120 gene transcription, and c) the position of Sp1 with respect to the transcription initiation site is important for regulation of p120 gene transcription.

Using HeLa nuclear extract, Gel retardations, footprint analyses, and competitions with oligos of known Sp1 sequences suggested that an Sp1 trans-acting factor interacts with the sequence AGAGGCGGGG (−425/−416) within the −537/−278 cis-acting region. The nucleotide G at position −422 is required for binding. The p120 Sp1 recognition sequence has been found to be similar to other well defined Sp1 binding sequences (Table 1, ref.24). Similar results were obtained with purified Sp1 factor obtained from Promega (data not shown). Deletion of the Sp1 binding sequence from the −2532/+98 CAT construct eliminated p120 gene transcription, demonstrating an essential role for Sp1 in the initiation of p120 gene transcription. The Sp1 binding sequence (−425/−416) also lies within the functionally important region −472/−400 which was found to stimulate a heterologus promoter CAT construct (TK CAT) about two fold (Fig.1). The 35 bp PRE sequence (−472/−437), when placed in both orientations just upstream of the Sp1 box, stimulated transcription about 2.4−3 fold, suggesting it has an enhancer function. This PRE region does not bind any protein factor (since deletion of the Sp1 binding site −429/−412 from the −537/−278 region eliminated any protein factor binding)

Table 1. Comparison of Sp1 binding sequences of p120 and other genes.

Sp1 binding sequence	Relative affinity	Gene
A G A G G C G G G G	medium	p120
T G G G C G G G G T	medium	HSV TK (II)
G G G G C G G G G T	medium	HSV 1E-3 (II)
T G G G C G G G G C	high	HSV IE-3 (III,IV)
G G G G C G G C G C	low	HSV-TK (I)

under the experimental conditions used. The PRE sequence is GC rich (25 GC bp out of 35 bp) and does not match with any other cis-acting sequences of other genes. The sequence −457/−429 is important in stimulating heterologous TK promoter activity (see Fig. 1). The sequence common to the PRE −472/−437 and the −457/−429 region extends from −457 to −437; this 20 bp sequence CCGGGTGCGGCGGCCGACTA may be the positive regulatory cis-acting sequence (PRE).

The Sp1 factor has an important role in transcription of many genes(21,23−28). There are differences with respect to the locations of the Sp1 binding sites from the transcription start sites in various genes. The Sp1 binding sites are usually in close proximity to the transcription initiation sites. In the case of the p120 gene, the absolute requirement of an Sp1 binding site −406 bp upstream is interesting.

Sequence-specific transcription factors may stimulate transcription by interacting with each other (27,29,30,), possibly because specific DNA binding factors are brought in close proximity by a looping out of the DNA segments, which facilitates interaction of factors (29,31). With respect to p120 gene transcription, the distance of the Sp1 binding site with respect to the initiation site is very important for efficient transcription (see Fig 5); the sequence −406/−278 can be deleted and substituted by a random sequence of the same length without any appreciable effect on p120 gene transcription (Fig 5), which suggests this segment of DNA acts as a 'spacer'.

Since Sp1 is absolutely essential for transcription of the p120 gene, perhaps Sp1 interacts with the transcription initiation complex. The 'spacer' sequence −406/−278, which is required for optimum transcription, may facilitate a looping-out of the Sp1 binding site, and may place Sp1 in close proximity to the transcription machinery.

Sp1 interaction with other factors has been suggested (21, 27, 30,32). Janson *et al.* (27) reported that the functional cooperativity between OTF-1 and Sp1 involves physical interaction between the two transcription factors in the case of U2 SnRNA maxi genes. Smale *et al.*(30) reported that a mammalian transcription factor, the TFIID protein fraction, was required for transcriptional stimulation by an Sp1-dependent activating element placed upstream of either TATA or initiator elements of synthetic genes. Pugh *et al* (33) showed that in a reconstituted system Sp1 activates transcription from TATA containing promoters by indirectly interacting with TFIID which is mediated by a co-activator present in the semipurified TFIID fractions from either human or Drosophila cells.

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