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Characterization of the P4 promoter region of the human insulin-like growth factor II gene

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The human insulin-like growth factor II (IGF-II) gene contains four promoters (P1, P2, P3 and P4). In order to determine the mechanism by which the P4 promoter is controlled, the human IGF-II P4 promoter was analyzed in cell lines. DNA sequence analysis of the human IGF-II P4 promoter gene showed that the P4 promoter region contains a TATA-like sequence and several G + C rich regions which are essential for transcription. Analysis of the transcription initiation site by S1 nuclease mapping revealed two transcription start sites; both are located immediately behind TATA-like sequence. To determine the location of sites that may be important for the function of the human IGF-II P4 promoter, we constructed chimeric genes of the human IGF-II P4 promoter fused to the coding region for chloramphenicol acetyltransferase (CAT). These constructs were transfected into HepG2, PLC/PRF/5, G401 and A549 cells, and were examined for CAT activity. All transfected cells showed a similar profile of CAT activity. Sequences responsible for putative enhancer and silencer regions were identified and the 5′ flanking sequences of the human IGF-II P4 promoter contain negative regulatory regions (–213 to –174). The 53-base pair fragment located between 111 and 59 base pairs upstream of the start site contains positive regulatory activity. Gel mobility shift assay showed that Sp1 and another proteins might be involved in positive regulation of the human IGF-II P4 promoter.

IGF-II P4 promotor; Positive regulatory element; Negative regulatory element; Sp1 binding sequence; Egr-1 element

1. INTRODUCTION

Insulin-like growth factor II (IGF-II) is a fetal growth factor which is known to act on fetal development and differentiation [1]. Although IGF-II is structurally related to insulin and IGF-I, its physiological function is not fully understood. The genomic structure of the IGF-II gene spans about 35 kb, which includes nine exons and four promoters [2–5]. Structural analysis of rat and human IGF-II cDNA clones and their genes reveal the presence of alternatively used leader exons which are responsible for the generation of multiple transcripts, in which they provide 5′-untranslated regions that are variable in length and sequence. Transcription of the human IGF-II gene yields 6 kinds of mRNA species which are expressed in a tissue-specific and development-specific manner. In human adult liver tissue, a 5.3 kb mRNA species is dominant, while two other species of mRNAs, 6.0 and 4.8 kb, are dominant in many fetal liver and non-hepatic tissues. Each of these IGF-II mRNAs is expressed under the control of 4 different promoters [6]. High levels of IGF-II mRNAs have been reported in many tumors and tumor cell lines including Wilms' tumors and rhabdomyosarcomas [7], whose expression is owed to promoters 3 and 4 preceding exons 5 and 6, respectively, which induces the expression in fetal liver. Also in primary liver cancers and some cirrhotic tissues surrounding liver cancer, fetal types of IGF-II transcripts are expressed [8].

To obtain more insight in the mechanisms involved in the regulation of the IGF-II gene expression, the IGF-II promoters have been characterized [5,9]. Promoters P3 and P4 are expressed in all fetal and in non-hepatic adult tissues, whereas in adult liver, the P2, P3 and P4 promoters are completely shut off and promoter P1 is activated [5]. P3 promoter is well characterized. This promoter contains the TATA box and four binding sites for nuclear proteins [9]. Recently, it has been reported that the P3 promoter contains three binding sites for the transcription factor Egr-1 (early growth response element), suggesting that these motifs are important for the basal expression of the human IGF-II gene under the control of the P3 promoter [10,11]. Studies on the human P4 promoter and its corresponding rat promoter (P3) of the IGF-II gene have recently been reported [12,13]. Among the findings was the identification of a distinct promoter region containing multiple Sp1-binding sequences and a TATA-like sequence. Even though human IGF-II P4 promoter was examined [13], the specific elements regulating the human P4 promoter were not characterized.

In this paper we report the characterization of the P4 promoter of the human IGF-II gene which regulates the transcription of the 4.8 kb mRNA in fetal liver. We have

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undertaken this study to increase our understanding of the important conserved regulatory elements critical for expression of human IGF-II under the control of the P4 promoter and to extend our promoter studies to different biological systems.

2. MATERIALS AND METHODS

2.1. *Nucleotide sequence determination and plasmid construction*

The fourth (P4) promoter of the human IGF II gene-CAT chimeric construct was generated as follows: plasmid pHIGF II-14-6 was digested with *Nru*I and *Bst*EII (−411 to +124). After the ends were filled in, the resulting fragment was inserted into *Sma*I site of the multiple cloning site of the pGEM-SVOCAT [22]. To sequence the *Nru*I/*Bst*EII (−411 to +124) fragment, a 540 bp insert was obtained from pIGO by digesting the plasmid with *Hin*dIII/*Kpn*I restriction enzymes which are present in the multiple cloning site, and subcloned into the vectors M13 mp 18 and 19. The nucleotide sequence was determined by the dideoxy chain termination method. The deletion mutant series of the P4 promoter of the human IGF-II gene was constructed by PCR amplification using oligonucleotides designed to generate ends with *Hin*dIII and *Xba*I restriction sites. The PCR product was then cloned into pGEM4-SVOCAT which was cut with the same enzymes. The constructed plasmids were purified in two sequential CsCl centrifugation steps for maximum activity.

2.2. *Cell culture, transfection and CAT assays*

Human hepatoma cells, PLC/PRF/5 and HepG2, were grown in minimal essential medium supplemented with 10% fetal bovine serum. Human lung adenocarcinoma (A549) cells were grown in Dulbecco's modified Eagle's medium with high glucose supplemented with 5% fetal bovine serum. Wilm's tumor (G401) cells were grown in McCoy's 5a medium with 10% fetal bovine serum.

Transfections were carried out by the calcium phosphate co-precipitation method [23]. Cells were transfected with 10 μg of plasmids. Briefly, the cells were incubated with the plasmid DNA for overnight and then exposed to 15% (v/v) glycerol in 20 mM HEPES buffer for 2 min. After glycerol shock, the cells were further incubated for 48 h in complete medium. The cells were then harvested and extracts were assayed for CAT activity according to the method of Gorman et al. [24]. CAT enzyme activity was normalized for transfection efficiency by co-transfection of 2.0 μg of the β-galatosidase expression plasmid pCH110 and measuring the β-galactosidase activity of the individual extracts of the transfected cells.

2.3. *Nuclease protection assay*

Total RNA was prepared from PLC/PRF/5 cells transfected transiently with the plasmid plG5 according to the method of Chomczynski and Sacchi [25]. The plasmid pIG5 was linearized with *Xba*I, dephosphorylated, end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase, and digested with *Hin*dIII. The resulting fragment (−111 to +129) was gel-purified and used as a probe. Total RNA (60 μg) was co-precipitated in ethanol with 1×10^6 c.p.m. of an end-labeled DNA probe representing the *Hin*dIII–*Xba*I fragment of plG5 (−111 to +129) (see Fig. 3). Hybridization was performed by dissolving the precipitate in 40 μl of hybridization buffer (80% formaldehyde, 20 mM Tris, pH 7.4, 0.4 M NaCl, 1 mM EDTA), heating for 15 min at 75°C and incubating for 16 h at 58°C. The DNA–RNA hybrid was diluted into 360 μl of S1 nuclease buffer (0.01 M NaCl, 0.1 mM $ZnSO_4$, and 2 mM sodium acetate, pH 4.5) containing 2 μg of denatured salmon sperm DNA and 150 units of S1 nuclease, and incubated at 37°C for 1 h. The protected fragments were recovered by ethanol precipitation, denatured, and analyzed on a 6% polyacrylamide gel.

2.4. *DNase I footprinting*

A DNA probe was prepared from the plasmid plG3 (Fig. 3) containing the promoter fragment from −174 to +129 cloned into the *Hin*dIII and *Xba*I site of the polylinker region. This fragment was labeled with [γ-^{32}P]ATP at the *Xba*I linker (+129) using T4 polynuclease kinase and re-cut at the *Hin*dIII site in the vector. The resulting 303 bp fragment was purified from the 6% native polyacrylamide gel. Nuclear extracts were prepared from PLC/PRF/5 cells as described by Osborn et al. [26]. DNase I foot-printing was performed by mixing 50 μg of nuclear extracts and 0.5–2 ng of purified DNA fragment per reaction according to the method of Angel et al. [27].

2.5. *Gel retardation assay*

Gel mobility shift assays were carried out as described [20] with some modifications. The constructed plasmids plG5, plG5-1, plG5-2, plG5-3 and plG6 were digested with *Hin*dIII, end-labeled with ^{32}P and then digested with *Sma*I. The ^{32}P-labeled promoter fragments were gel purified and each incubated with PLC/PRF/5 and A549 cell extracts. The DNA–protein complexes were analyzed on polyacrylamide gels[1]. Electrophoresis of DNA–protein complexes was carried out at 4°C in 5% polyacrylamide gels in 0.5 × TBE.

3. RESULTS

3.1. *Transcription initiation sites and analysis of the P4 promoter region of the human IGF-II gene*

Using *Hin*dIII and *Kpn*I restriction sites in plGO (Fig. 4), the 540 bp fragment containing the human IGF-II P4 promoter was subcloned into the vectors M13 mp18 and 19, and its sequence was determined (Fig. 1). The sequence includes 411 nucleotides upstream and 129 nucleotides downstream of the exon 6 of the human IGF-II gene. The deletion mutant series of the human IGF-II P4 promoter gene were constructed by PCR amplification using oligonucleotides designed to generate ends with *Hin*dIII and *Xba*I restriction sites. The PCR product was then cloned into pGEM4-SVOCAT which was cut with the same enzymes. The transcriptional start sites were located by S1 nuclease protection analysis using a 240-bp *Hin*dIII/*Xba*I fragment (plG5) extending from the nucleotide −111 to the nucleotide +129 (see Fig. 1). This fragment was end-labeled at the 5′ terminus of the *Xba*I site prior to digestion with *Hin*dIII and subsequently isolated from the agarose gels. Using this probe we were able to observe two S1 nuclease-resistant fragments after hybridization with PLC/PRF/5 mRNA transfected with plG3 plasmid (Fig. 2). S1 analysis showed that transcriptional start sites were located just behind the TATA-like sequence and were only two nucleotides apart. Their locations are 20 or 29 bp upstream compared with rat IGF-II exon 3 which is equivalent to human IGF-II exon 6 [14,15].

DNase I footprinting assay showed three protected regions when the PLC/PRF/5 nuclear extracts were mixed with labeled plG3 fragment (−174 to +129, Fig. 3). Protected regions a and b are G + C rich, suggesting that Sp1 may bind to these regions. The third protected

[1]The human Sp1 protein was obtained from Promega. The Sp1 competitors consisted of the 22 bp fragments; 5′-ATTCGAT CGGGGCGGGGCGAGC-3′. The AP1, AP2 and CREB competitor fragments were 5′-CGCTTGATGAGTCAGCCGGAA-3′, 5′- GATCGAACTGACCGCCCGCGGCCCGT-3′, and 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′, respectively.

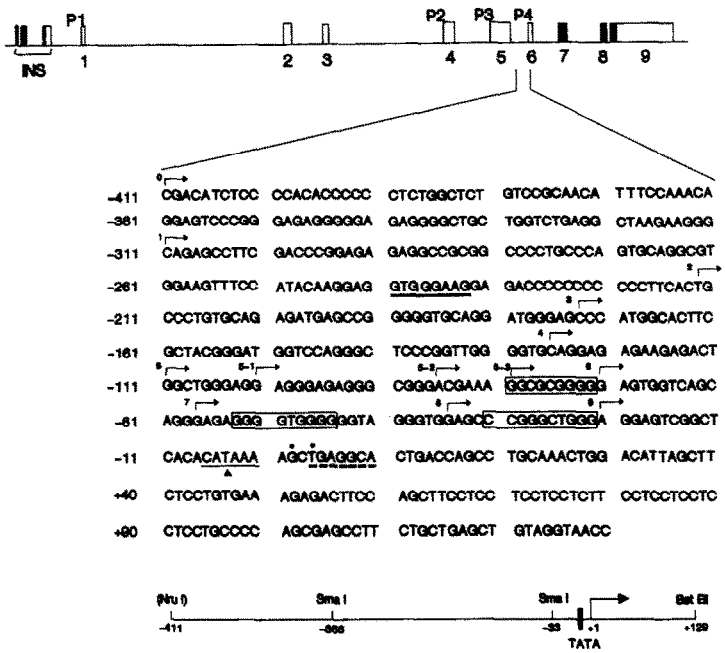

Fig. 1. Schematic presentation of the human IGF-II gene region and nucleotide sequence and restriction map of the 5'-flanking region (P4 promoter) of exon 6. Exons are represented by boxes and coding regions of the gene are shown by black boxes. The 540 bp *Nru*I–*Bst*EII fragment which contains the hIGF-II P4 promoter region was subcloned into pGEM4-SVO CAT, and the insert was cloned into the M13 mp18 and 19 vectors. The sequence was determined by the dideoxy chain termination method. Several potential transcription factor binding sites were analyzed through computer-aided search. Transcription initiation sites were determined by S1 nuclease analysis. The 5'-ends of serially deleted promoter mutants are indicated as numbers 0 to 9. Potential AP1 [16], Sp1 [27] and C/EBP binding sites are shown by broken underlining, open boxes and thick solid underlining, respectively. Closed circles and the closed triangle indicate the transcription start sites and TATA box, respectively.

region c is the TATA-like sequence and upstream sequences, which might be covered by a TATA box-binding protein.

Examination of the sequences showed several potential transcriptional factor binding sequences, including C/EBP at −241, AP1 at +3, and Sp1 at −84, −54 and −32 (Fig. 1). However, our data showed that C/EBP and AP1 binding sequences were not likely to play a significant role in the transcriptional regulation of the human IGF-II P4 promoter (Fig. 5, 7).

3.2. *Putative negative and positive regulatory regions in the P4 promoter of the human IGF-II gene*

To find out the regulatory elements of the P4 promoter of the human IGF-II gene, various portions of the promoter region deleted from the 5'-end were fused to the coding region of the bacterial chloramphenicol acetyltransferase (CAT) gene in the pGEM4-SVOCAT vector (Fig. 4). These constructs were transfected into HepG2, PLC/PRF/5, G401 and A549 cells. Fig. 5 shows the basal CAT activity in the several transfected cell lines. Deletion of the fragment from −213 to −174 increases the CAT activity in all cell lines except A549, while deletions from −111 to −59 sharply decreases CAT activity in all cell lines tested. This result suggests that the sequence from −213 to −174 contains a negative regulatory element and the sequence from −111 to −59 contains a positive regulatory element. In A-549 cells, CAT activity was increased when the region between nucleotide −174 and nucleotide −127 is deleted. Deletion of the fragment from −58 to −37 slightly increases CAT activity while further deletion to −22 slightly decreases CAT activity in all cell lines tested except A-549 cells. Taken together, these data suggest that the 5'-flanking sequences of the fourth promoter of the human IGF-II gene contain two negative regulatory regions (−213 to −174 and −174 to −127) which repress the transcription unit very efficiently, depending on the cell types.

3.3. *Identification of nucleoproteins bound to the P4 promoter region of the human IGF-II gene*

Gel mobility shift assays were carried out with ^{32}P-labeled P4 promoter fragments to identify the proteins which might be involved in the transcriptional regulation of the human IGF-II gene. Since deletion analysis showed that the sequences between −111 and −59 were responsible for positive regulation, we attempted to

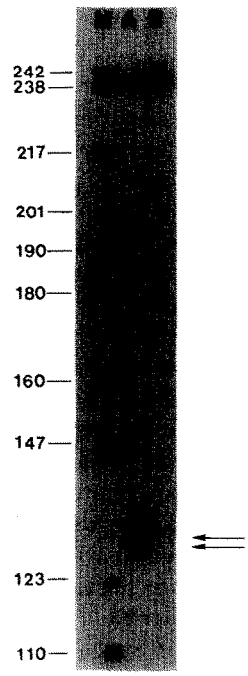

Fig. 2. Nuclease protection assay. PLC/PRF/5 cell mRNA was analyzed by S1 nuclease protection assay with the *Hin*dIII–*Xba*I fragment labeled at the 5' end of the *Xba*I site as described in Section 2. Lane A, total RNA from PLC/PRF/5 plus probe, protected fragments of 127 and 129 bp are shown; lane B, aliquot of probe. S1 protected fragments were analyzed by electrophoresis on a 5% polyacrylamide sequencing gel containing 8 M urea. The size of the protected fragment was determined by co-electrophoresis of the sequencing samples of the human IGF-II gene fragment. Lane M, molecular weight marker (pBR322 DNA + *Msp*I).

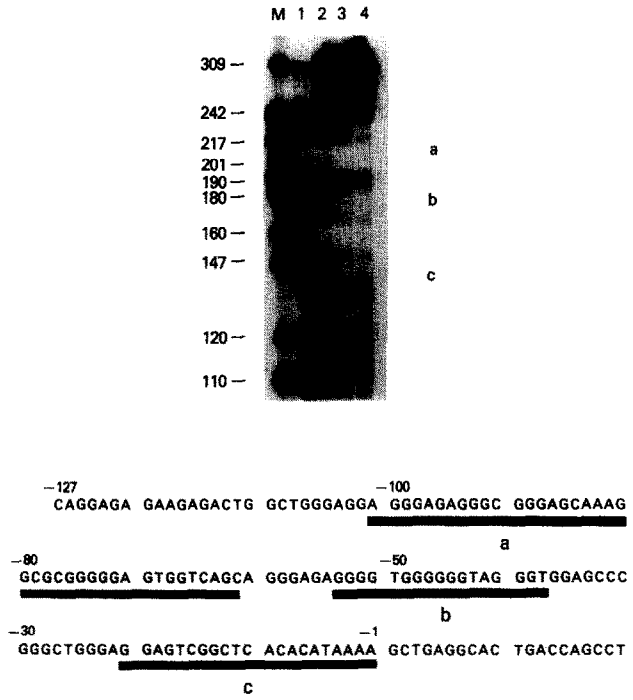

Fig. 3. Identification of protein binding region in the P4 promoter by DNase I footprinting. A DNA fragment containing 303 bp of the P4 promoter region from position −174 (*Hin*dIII) to +129 (*Xba*I) was prepared as probe and end-labeled with ^{32}P at the *Xba*I site. The probe was incubated with 25 μg of bovine serum albumin (lane A), or 5 (lane B), 10 (lane C) or 15 μl (lane D) of PLC/PRF/5 nuclear extracts. Three protected regions are designated as a, b and c. Lane M, molecular weight marker (pBR322 DNA + *Msp*I).

characterize the proteins binding to these regions. For this purpose, five fragments were prepared from the plasmids plG5, plG5-1, plG5-2, plG5-3 and plG6 (−111 to −31, −102 to −31, −87 to −31, −81 to −31 and −72 to −31, respectively) by digesting each plasmid with *Hin*dIII and *Sma*I restriction enzymes, and end-labeled as described in Section 2. These probes were separately incubated with nuclear extracts isolated from A549 cells, and electrophoresed on native polyacrylamide gels. There were several bands showing protein bindings, which disappeared in the presence of 100-fold molar excess of unlabeled fragments (Fig. 6 and unpublished data). Three major retarded bands were designated as a, b, and c. Complexes a and c disappeared when the deletion reached to −72. To confirm whether these binding proteins are related to the known DNA binding proteins, we carried out competition assays using oligonucleotides of Sp1, AP1, AP2, and CREB binding consensus sequences in gel mobility shift assays. When a 100-fold molar excess of Sp1 oligonucleotide was added, two major bands (a and b) disappeared, but not two minor bands (c and d), suggesting that the top two bands are the specific bands representing Sp1 binding (Fig. 7A, lanes 3 and 4). This result shows that Sp1 and another protein are involved in the positive transcriptional regulation of the P4 promoter. On the other hand, addition of a 100-fold molar excess of each AP1, AP2 and CREB oligonucleotide did not show any effect to the bands at all (Fig. 7A, lanes 5, 6 and 7). And purified Sp1 protein was used for a gel mobility shift assay using the plG5 fragment (−111 to −31) as probe (Fig. 7B). The consensus Sp1 oligonucleotide (lane 2) was able to compete nearly all of the binding of Sp1 to the P4 promoter fragment of the human IGF-II gene.

4. DISCUSSION

In both human and rat, all of the mRNA transcripts of the IGF-II gene exhibit tissue- and development-specific regulation, and transcription of each transcript is controlled by separate promoters. The P4 promoter of the human IGF-II gene corresponds to the P3 promoter of the rat IGF-II gene, and they show nearly 75% homology in nucleotide sequence [14,15]. The P4 promoter regulates the transcription of the 4.8 kb mRNA at the fetal stage in the liver. In this report, we have characterized several regulatory elements of the P4 promoter of the human IGF-II gene and regulatory proteins related to it.

Although the human IGF-II P4 promoter and its corresponding rat promoter (P3) appear well conserved

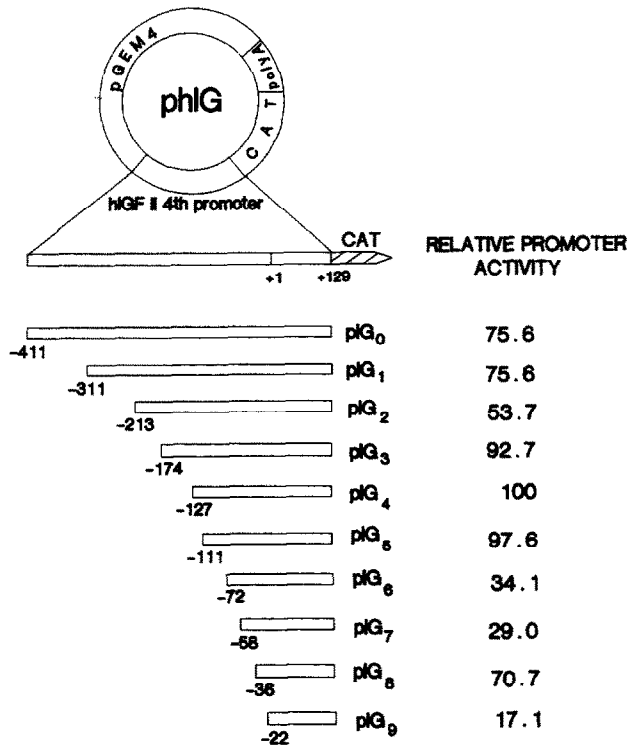

Fig. 4. Structure of P4 promoter of hIGF-II gene-CAT chimeric genes. The construct containing upstream sequences up to nucleotide −411 of exon 6 of the human IGF-II gene was used to construct deletion mutant plasmids, plGO to plG9. The solid bars represent various portions of the P4 promoter region produced by PCR amplification. The first transcription initiation site identified by S1 mapping is indicated as +1. The column on the right shows relative CAT enzyme activity obtained in several experiments with each plasmid in PLC/PRF/5 cells. Transfection efficiency was normalized as described in Section 2.

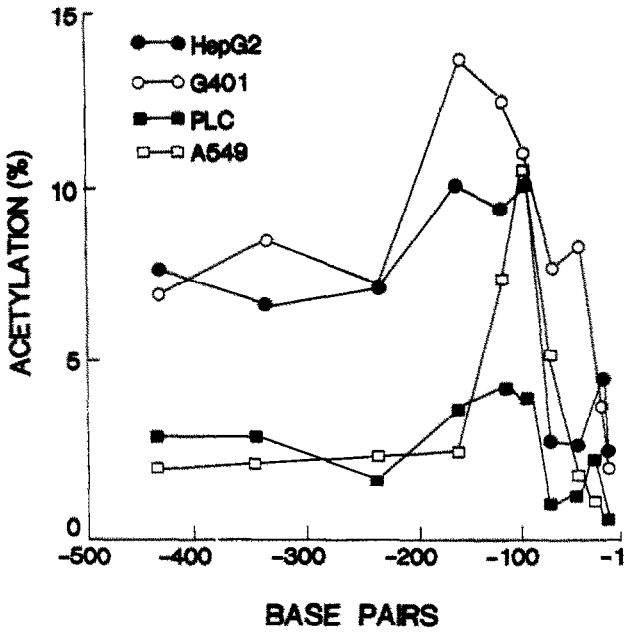

Fig. 5. Expression of 5'-deletion mutants of human IGF II P4 promoter-CAT fusion genes. The cells were transiently transfected with the pIG DNA series into HepG2, PLC/PRF/5, G401 and A549 cells. The positions of the 5'-ends of deletion mutants are described in Figs. 1 and 4. The cells examined here exhibit similar profiles of transcriptional activities. Transfection efficiency was normalized as described in Section 2. Each experiment was repeated at least three times.

in sequence and in general structure, the transcriptional initiation site in the human IGF-II gene under the control of the P4 promoter was identified 2 nucleotides upstream relative to that of the rat start site [12]. Our S1 analysis of the total RNA prepared from the PLC/PRF/5 cells transfected with the plasmid plG3 indicated that the transcriptional start site is also different from the previously reported one [13]. This may be due to the fact that we have prepared the total RNA from the PLC/PRF/5 cells transfected with the plasmid plG3.

We examined the basal expression of the P4 promoter deletion mutants–CAT chimeric constructs in several cell lines. We identified that the human IGF-II P4 promoter contains both negative and positive regulatory elements. Deletion of the negative regulatory region (−213 to −174) resulted in a increase of the transcriptional activity of the promoter, while removal of the putative positive regulatory region (−111 to −59) sharply decreased the transcriptional activity of the human IGF-II P4 promoter (Fig. 5). The region between −111 and −59 was also positively regulated by the protein phosphatase inhibitor okadaic acid, a non-TPA-type tumor promoter (manuscript submitted). The more detailed analysis revealed that the Egr-1 element, located between nucleotide −78 and −70, is responsible for the induction of P4 promoter activity by okadaic acid. Since the deletion from −111 to −59 sharply decreases CAT activity, it is likely that the Egr-1 element is the major transcription factor responsible for the basal expression of human IGF-II under the control of the P4 promoter. These results suggests that for maximum expression of the promoter, at least 111 base pairs upstream from the transcription initiation site are required.

Analysis of the human IGF-II P4 promoter has shown several G + C rich regions which are essential for transcription [18–20]. In particular, there are three possible Sp1 binding sequences within 100 bp upstream from the transcription start site, which are clustered in the positive regulatory region. Incubation of the plG5 fragment with A549 nuclear extracts in the presence of Sp1 as specific competitor resulted in the disappearance of the bands a and b (Fig. 7A), which suggests that Sp1 binds to the P4 promoter and is involved in the transcription of human IGF-II gene.

Sequence analysis also revealed one possible AP1 binding sequence located at just behind the transcription start site. However, the DNase I protection assay did not show any protein binding to this region (Fig. 3) and gel mobility shift assay with AP1 oligonucleotide as competitor also did not show any interference to the DNA–protein interaction (Fig. 7A). The binding sequence of another putative transcription factor, C/EBP, is located at −241. C/EBP is known to function as a

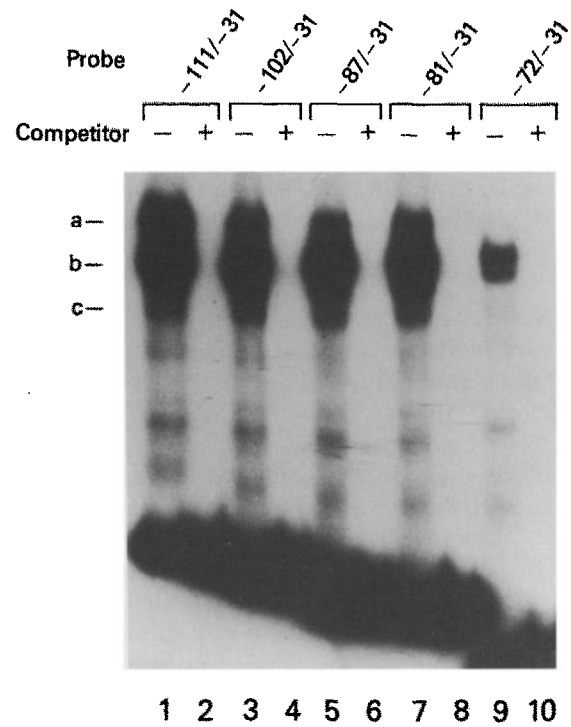

Fig. 6. Identification of specific binding of proteins to the human IGF II P4 promoter region. Five DNA fragments from plG5 (−111 to −31), plG5-1 (−102 to −31), plG5-2 (−87 to −31), plG5-3 (−81 to −31) and plG6 (−72 to −31) were each end-labeled at the *Hin*dIII site and purified for probes. Each probe was incubated with PLC/PRF/5 cell extracts in the presence (+) or absence (−) of specific competitor. The bands resulting from the specific binding of proteins to plG5 fragment are shown. In all cases, the bands disappeared when 100-fold molar excess of competitors were added.

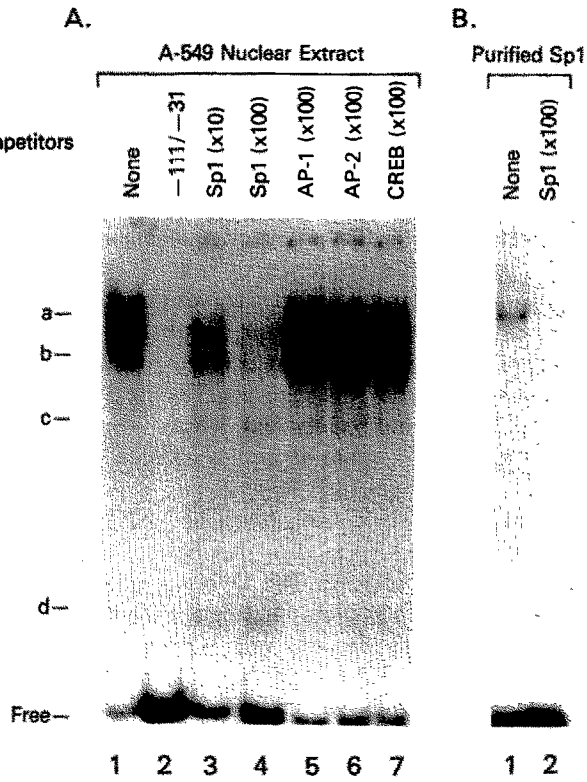

Fig. 7. Detection of cellular factors that bind to the human IGF-II P4 promoter. (a) The pIG5 fragment (−111 to −31) was incubated with a nuclear extracts from A549 cells in the presence or absence of a specific competitor and the DNA–protein complexes were gel electrophoresed. Two major retarded bands are visualized (lane 1). Binding was competed with 100-fold molar excess of unlabeled pIG5 fragment (lane 2) and synthetic oligonucleotide corresponding to the Sp1 binding consensus sequences (lane 3 and 4), respectively. (b) Sp1 binds directly to the P4 promoter of the human IGF-II gene. Binding reactions with 15 ng/μl pure Sp1 (Promega) were done as described in Section 2.

positive transcription factor for the thymidine kinase promoter of herpes virus and, on the other hand, functions as a transcriptional repressor for SV40 and hepatitis B virus (HBV) [21]. However, removal of the potential C/EBP binding sequence did not show any significant effect on CAT activity (Fig. 5). This result suggests that C/EBP binding per se may not be sufficient to induce activation or repression in the P4 promoter of the IGF-II gene as in the case of the promoter of RSV LTR [21]. The identification of the proteins binding to the negative regulatory elements is in progress. Further experiments on the DNA binding proteins will clarify the regulatory mechanism governing the expression of the human IGF-II P4 promoter.

The data presented here indicate that the general structure of the human IGF-II P4 promoter is similar to the human IGF-II P3 promoter [11]. Transcription factors suggested to play a role in the regulation of both P3 and P4 promoters include the transcription factors Sp1 and Egr-1. Further characterization of the role of these transcription factors in the regulation of IGF-II expression will provide further insight into understanding the role of IGF-II during development and carcinogenesis.

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