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Transactivation of mouse insulin-like growth factor II (IGF-II) gene promoters by the AP-1 complex

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ABSTRACT

The mouse insulin-like growth factor II gene (*Igf2*) is transcribed from three promoters (P1, P2 and P3), and is expressed in a tissue-specific and developmentally regulated fashion; however, little information is available on the transcription factors controlling *Igf2* expression. The AP-1 complex is a transcription factor involved in the regulation of a variety of genes, including those encoding certain growth factors. We show that *Igf2* P3 is transactivated by AP-1 in a transient expression assay, and that this effect is mediated through two non-consensus AP-1 binding sites characterised by DNA–protein interaction studies. Mutational analysis indicates these sites are required for AP-1 responsiveness and full promoter activity.

INTRODUCTION

The insulin-like growth factors (IGF-I and IGF-II) are mitogenic polypeptides displaying structural similarities to proinsulin (1,2). IGF-II is required for normal pre-natal growth in the rodent (3) and is encoded by a complex transcriptional locus, comprising three promoters and multiple polyadenylation signals (4,5). In the rodent embryo, IGF-II gene transcripts can be detected in a variety of tissues and offer an example of temporal and spatial regulation of gene expression with transcript levels declining after birth in most tissues (6, 7, 8). There is as yet little information available regarding transcription factors which address the IGF-II gene promoters. Promoters of the human gene have been most extensively characterized (9, 10, 11, 12). Drummond *et al* (13) have recently reported a repression of human IGF-II promoter 3 (similar in sequence to promoter 2 of the rodent gene) by the product of the Wilms' tumour susceptibility gene. Studies carried out on promoter 3 (P3) from the rat gene (14, 15) indicate that this promoter possesses a compact structure, requiring for maximal activity no more than 128 bases upstream of exon 3, including a TATA box and binding sites for the Sp1 transcription factor.

In this paper we describe the transactivation of *Igf2* promoters by AP-1 in a transient expression system using CP-1 embryonic stem (ES) cells, which present low endogenous levels of *Igf2* and AP-1 binding activity. DNA–protein interaction studies demonstrate the presence of two AP-1 binding sites in the proximal promoter region and, following their mutation, they were found to be necessary for AP-1 transactivation and full promoter activity. During embryonal carcinoma (EC) and embryonic stem (ES) cell differentiation *Igf2* and c-jun mRNA levels, and AP-1 binding activity, have been shown to increase (16, 17, 18 and this paper), raising the possibility that AP-1 might be involved in the induction of *Igf2* expression in this system.

MATERIALS AND METHODS

Cells

Buffalo Rat Liver cells (BRL cells; 19) were grown at 37°C, 5% CO_2 in alpha-Ham's medium (Gibco-BRL) supplemented with 10% fetal calf serum (Hyclone), ampicillin (75mg/l), streptomycin (50mg/l) and 50mM β-mercaptoethanol. BRL conditioned medium was harvested every three days and employed in the preparation of the ES cell culture medium. Mouse CP-1 ES cells (20) were grown in a 7:3 (vol:vol) ratio of BRL conditioned medium to alpha-Ham's medium (supplemented as above), at 37°C, 5% CO_2 (21). CP1 cells utilised for differentiation studies were cultured in the absence of BRL conditioned medium for 10 days prior to use. Differentiation was monitored by checking the levels of the stage-specific surface antigens SSEA-1, SSEA-4 and laminin. Although CP-1 cells cultured in the presence of BRL CM did not display a significant SSEA-1-positive reaction, they proved to be negative for laminin expression; low levels of SSEA-4 antigen were also detected (up to 10% of the cells). Withdrawal of BRL CM from the medium for 10 days resulted in an increase in the number of SSEA-4-positive cells (up to 20% of the cells) and laminin-positive cells (up to 30%).

Plasmids

Unless otherwise stated, DNA manipulations were performed using techniques essentially as described in Sambrook, Fritsch and Maniatis (22). Promoters of the mouse IGF-II gene were isolated from the clone cosIGF4 (23) and reporter fusions were

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created in the pLUC and pLUCS vectors, containing the firefly (*Photinus pyralis*) luciferase coding region and the SV40 small t intron and polyadenylation signal (24; Figure 3a). All mouse IGF-II gene nucleotide positions refer to transcript initiation sites defined by Rotwein and Hall (23). Mouse *Igf2* promoter P1 was isolated as a 2.2 Kb *Bam*HI fragment including the two 5′-most transcriptional start sites of exon 1, and cloned into pLUC, giving rise to pP1. P2 and P3 were sub-cloned from cosIGF4 within a 5.6 kb *Bam*HI fragment (from nucleotide position −42 5′ of the third transcriptional initiation site in exon 1, to nucleotide position +11 within exon 4) and inserted into pBluescriptSK+ (Stratagene) to form pP2P3. P2L was constructed by inserting into pLUC a 2.0kb *Pst*I fragment from pP2P3 utilising sites in the polylinker sequence 5′ of the insert and at position +383 in exon 2. For the construction of luciferase fusions with P3 sequences, the *Igf2* translational initiation codon (present in exon 4 at position +7) was eliminated by sub-cloning the insert from pP2P3 into pUC19 (Pharmacia) as a *Kpn*I fragment, utilising sites within the polylinker of pBluescriptSK+, 5′ of the pP2P3 insert and within exon 4 (from position −1 to position +5). This step created the clone pKP2P3. pP3L was derived from pKP2P3 by cloning into pLUC a *Pst*I−*Bam*HI fragment comprising sequences between the *Pst*I site at position +383 in exon 2 and the *Bam*HI site in the polylinker sequence 3′ of the insert. pP3M is a derivative of pP3L, lacking all *Igf2* genomic sequences upstream of the *Nhe*I site at position −162 5′ of exon 3. To generate pP3MM and pWTluc an *Nhe*I−*Bgl*I (−162 to +74 with respect to exon 3) fragment was rendered blunt-ended with the Klenow fragment of *E.coli* DNA polymerase I and cloned into a deletion derivative of pBluescriptSK+ (lacking polylinker sequences from the *Xba*I site to the *Xho*I site) giving rise to the intermediate clone pBstP3, from which it could be recovered as a *Xba*I−*Xho*I fragment including only 22bp of polylinker sequence (17 base pairs 5′ and 5 base pairs 3′ of the insert). The *Xho*I−*Xba*I fragment was then also rendered blunt-ended and ligated either into pLUC (at the *Bam*HI site rendered blunt-ended) or into pLUCS (at the *Stu*I site), generating pP3MM and pWTluc respectively. Mutagenesis of the two AP-1 sites was achieved by PCR, utilising pBstP3 as a template. For each site, 2 primer pairs were used (lower case indicates substituted bases): for site 1 (nucleotide position −39 to −29 with respect to exon 3) pair a: M13 reverse sequencing primer (5′AACAGCTATGACCA-TG3′) and MIGFP3-31 (5′gggGACATAAAAAGCGGAGG3′); pair b: M13 −20mer (5′GTAAAACGACGGCCAGT3′) and M-IGFP3-34 (5′GgGGCTCCTCCCAGTCCCGG3′). For site 2 (nucleotide position −92 to −80) primer pair c: M13 reverse sequencing primer and MIGFP3-35 (5′cCtGCAGGGAGGGG-GTGGGGGGTA3′); pair d: M13 −20mer and MIGFP3-36 (5′CCtCTCCCCCCGCCCCCTTT3′). Each PCR amplified fragment was sub-cloned into pBluescriptSK+. The insert pairs indicated were then ligated together to generate derivatives of pBstP3 lacking AP-1 sites (pPCR1, with mutations within site 1 generating a *Sma*I site; pPCR2, with mutations within site 2 generating a *Stu*I site). The generation of a pBstP3 version lacking both AP-1 sites (pPCR3, containing a *Sma*I site and a *Stu*I site) was achieved by using pPCR1 as a template and primer pairs c and d for the PCR reactions, followed by sub-cloning as for pPCR1 and pPCR2. The inserts from pPCR1, pPCR2 and pPCR3 were excised as *Xba*I−*Xho*I fragments, rendered blunt-ended and sub-cloned into the *Stu*I site of pLUCS generating transcriptional fusions (pPCR1luc, pPCR2luc and pPCR3luc) identical to pWTluc except for the desired mutations (as determined by DNA sequencing).

The mouse c-*jun* and c-*fos* expression vectors have been reported previously (25) and were provided by Dr Michael Karin (University of California, San Diego). The pSV2ALΔ5′, pSV0ALΔ5′ (26: in this paper abbreviated to pSV2 and pSV0 respectively) and pIRV-Neo-Act-LacZ (27; referred to in this paper as pIRV) plasmids were provided by Dr Suresh Subramani (University of California, San Diego) and Dr Rosa Beddington (University of Edinburgh). The mouse *Igf2* and GAPDH cDNAs (28, 29) employed to probe Northern blots were supplied by Dr Graeme Bell (Chiron Corporation, California), and Dr J.K.Heath (Oxford, UK).

RNA isolation and analysis

Approximately 3μg samples of polyadenylated RNA (30, 31) were electrophoresed in formaldehyde/1% agarose gels (22) and blotted onto nitrocellulose filters (Schleicher & Schuell). Filters were hybridized with probes comprising mouse *Igf2* and GAPDH cDNA sequences (28, 29) labelled with α[^{32}P]dCTP (3000Ci/mM) to a specific activity of approximately 5×10^8cpm/μg DNA.

Transient expression assays

Cell transfections were performed by the electroporation technique. Cells were dispensed into disposable gene pulser cuvettes (Biorad, 0.4 cm electrode gap) in 400μl aliquots at a concentration of approximately 1.2×10^6 cells/ml; plasmid DNAs, resuspended in serum-free alpha-Ham's medium at a concentration of 1mg/ml were added as indicated in the results section. In most experiments, the total amount of DNA added to each sample was adjusted to 70μg with pBluescriptSK+, and the final volume in each cuvette was adjusted to 500μl with serum-free alpha-Ham's medium. For direct comparisons between pWTluc and pPCR3luc constructs, cells were transfected with 20μg of either construct and 10μg pIRV. Electroporation was carried out using a Biorad Gene Pulser, equipped with capacitance extender, at 250V, 500μFD. After a 15 minute incubation at 20°C, cells were transferred to 9ml serum supplemented medium in 10cm diameter tissue culture dishes and cultured for 12 hours. This time point was found to be optimal following electroporation of promoter-luciferase constructs in preliminary experiments (data not shown).

Luciferase and β-galactosidase enzyme activities were measured with an Autolumat LB 953 luminometer (Berthold) and Titertek multiscan MCC/340 spectrophotometer respectively, using reagent kits supplied by Promega (following manufacturer's instructions). Amounts of soluble protein contained in cell lysates were measured spectrophotometrically using the Protein Assay Dye reagent (Biorad) as per manufacturer's instructions. Luciferase readings were adjusted relative to protein levels and to β-galactosidase activity in order to control for variations in transfection efficiency and cell number. Background readings obtained from control samples (cells electroporated in the absence of plasmid DNAs) were subtracted from the readings obtained from experimental samples (cells electroporated in the presence of *Igf2* promoter-luciferase constructs). Transient expression experiments were repeated at least three times, with all samples being treated in duplicate on each occasion.

DNA gel-shift assays

Preparation of nuclear extracts was according to Andrews and Faller (32). 5μg of nuclear proteins were incubated with 7μg polydIdC (Sigma) in 25mM Tris−HCl (pH 8), 50mM KCl, 6.25mM $MgCl_2$, 0.5mM EDTA, 10% glycerol, 0.5mM DTT for 10 minutes at 20°C in a final volume of 30μl; a 50-fold excess of unlabelled competitor oligonucleotide, comprising either an AP-1 specific oligonucleotide (5′CGCTTGATGAGTCAGCCGGAA3′, Promega) or an Sp1 specific (control) oligonucleotide (5′ATTCGATCGGGGCGGGGCGAGC3′, Promega) was added. Probe, 0.1−1ng AP-1-specific oligonucleotide end-labelled with γ[^{32}P]dATP (3000Ci/mM, NEN-Dupont) to a specific activity of $5-10\times10^7$cpm/μg, was then added and incubation was continued for a further 10 minutes prior to gel loading. DNA−protein complexes were separated on 8% nondenaturing polyacrylamide gels poured and run in 1×TBE buffer (Tris−Borate−EDTA, pH 8).

DNAse I footprinting

Footprinting reactions were performed using purified human c-jun protein (Promega) according to manufacturer's instructions, except that binding reactions were carried out at 20°C for 10 minutes. Following protein binding, 1−5ng of the probes (either pBstP3, singly labeled at a *Xho*I site or the SV40 early promoter-enhancer region, singly labelled at an *Eco*RI site, end-labelled to a specific activity of $2-4\times10^6$ cpm/μg) were subjected to DNAse I digestion using 0.02 units of DNAse I (Promega) for 1 minute at 20°C in a 50μl reaction volume. Reactions were terminated by addition of 2× stop buffer (0.2M NaCl, 0.03M EDTA, 1% SDS, 100μg/ml yeast tRNA), followed by phenol-chloroform (phenol:chloroform:isoamyl-alcohol; 24:24:1) extraction and ethanol precipitation. DNA pellets were resuspended 1:1 (v/v) in water:loading dye (loading dye: 0.1 M NaOH/formamide 1:2 v/v, 0.1% xylene cyanol, 0.1% bromophenol blue) and separated on 6% denaturing polyacrylamide gels. Footprinted regions were analyzed by comparison with DNA sequence obtained with Sequenase 2 reagents (United States Biochemical), using pBstP3 as a template and priming the reaction with a primer (5′TCGAGGGGCTGGAAGAGG3′) in which the 5′-most base was the same as the end-labelled base of the probe (pBstP3) used in *Igf2* P3 footprinting reactions.

RESULTS

AP-1 activity and *Igf2* transcript levels are induced upon CP-1 cell differentiation

In order to monitor AP-1 activity, nuclear extracts were prepared from CP-1 ES cells cultured in the presence and absence of BRL cell conditioned medium. 5μg of nuclear proteins from each extract were employed in gelshifts utilising a double stranded oligonucleotide containing the AP-1 recognition sequence (TGACTCA, Figure 1). A substantial increase in AP-1 binding activity was observed upon CP-1 ES cell differentiation (Figure 1, lanes 2 and 3). Competition with unlabelled AP-1 specific (lane 4) and Sp1 specific oligonucleotides (lane 5) demonstrates the specificity of binding. We note that in some experiments, as in the example presented here, some competition by the Sp1-specific oligonucleotide was observed. However, experiments using various amounts of competitor oligonucleotides demonstrate that an AP-1-specific competitor was effective at much lower concentrations than competitor oligonuclotides bearing consensus binding sites for Sp1, Oct-1, and the glucocorticoid receptor (data not shown). These results are consistent with those reported previously by de Groot *et al* (17) following retinoic acid-induced differentiation of ES cells.

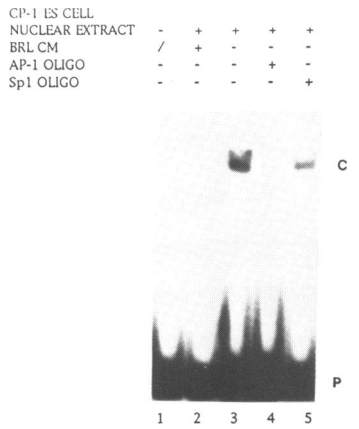

Figure 1. Gelshift obtained from nuclear extracts of CP1 ES cells using an AP-1-specific oligonucleotide. Extracts were from cells grown in the presence (lane 2) or absence (lanes 3, 4 and 5) of BRL cell-conditioned medium. All samples contained a radiolabelled AP-1 specific oligonucleotide (probe); unlabelled competitor oligonucleotides were present in samples 4 (AP-1-specific) and 5 (Sp1-specific). Positions of free (P) and complexed (C) probe are shown. Lane 1 represents the blank (probe oligonucleotide alone).

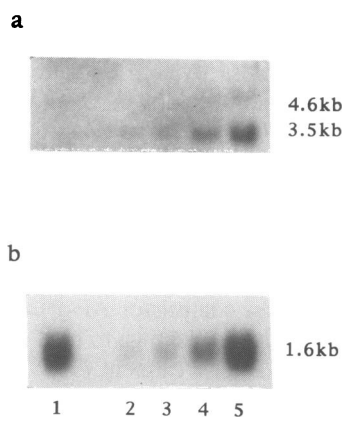

Figure 2. Northern blot of polyadenylated RNA samples from CP-1 cells cultured in the presence (lane 1 contains 3 μg RNA) and absence (lanes 2−5 contain 0.2, 0.5, 1, and 3 μg RNA, respectively) of BRL cell conditioned medium. **a.** The blot was hybridized with *Igf2* cDNA sequences. The signals at 3.5kb and 4.6kb represent *Igf2* transcripts of P3 and P2 origin, respectively. **b.** The same blot was reprobed with a GAPDH cDNA fragment to control for RNA loading.

Igf2 transcript levels were found to rise upon CP-1 ES cell differentiation (Figure 2), as previously shown following differentiation of EC and ES cell lines (18).

Transactivation of *Igf2* promoters by AP-1 in CP-1 cells

To investigate a possible effect of AP-1 on IGF-II gene expression, genomic fragments encompassing the IGF-II gene

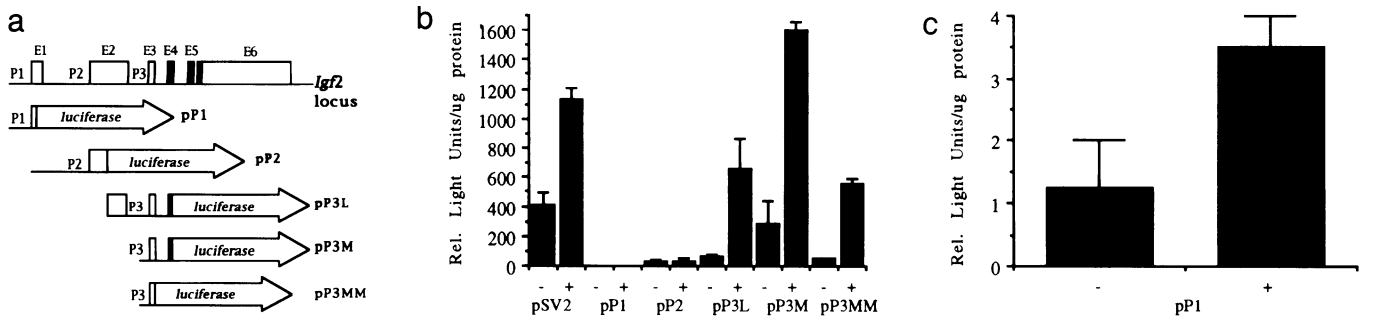

Figure 3. a. Structure of the mouse *Igf2* locus, and promoter-luciferase fusion constructs. The locus is composed of six exons (E1 to E6, of which only the solid black regions of exons 4, 5 and 6 code for protein) and three promoters (P1, P2 and P3). The luciferase coding region is represented by arrows. **b.** Effects of AP-1 on *Igf2* promoter-luciferase constructs introduced transiently into CP-1 ES cells (the pSV2 construct was employed as positive control). Luciferase activity obtained in the absence (−) and presence (+) of c-*jun* and c-*fos* expression constructs are given. The means and standard errors of values obtained in one representative experiment are given. **c.** The activity of pP1 was very low relative to the activity of other constructs, and is shown separately.

promoters were isolated from the mouse cosmid clone cosIGF4 (23). The constructs pP1, pP2 and pP3L produced luciferase in BRL cells, which have been previously shown to express abundant levels of IGF-II transcripts (6), and did not do so when the orientation of the *Igf2* sequences with respect to the luciferase coding region was reversed (data not shown). Each *Igf2* promoter-luciferase construct (Figure 3a) was then tested in a transient expression assay for transactivation by cotransfected mouse c-*jun* and c-*fos* expression vectors in CP-1 cells, which have relatively low levels of *Igf2* transcripts (Figure 2) and endogenous AP-1 activity (Figure 1). An induction of luciferase activity in the presence of c-*jun* and c-*fos* expression vectors was observed for pP3 (up to 15-fold, Figure 3b), and pP1 constructs (2 to 3-fold, Figure 3c). The pSV2 construct (26), containing the luciferase coding region driven by the SV40 early promoter-enhancer, was used as a control for AP-1 transactivation; this promoter has previously been shown to bind AP-1 (33, 34) and to respond to phorbol ester treatment (35).

Further characterisation of the effect of AP-1 on *Igf2* expression focussed on the elements contained in P3 which had shown the strongest response. By assaying progressively smaller regions of the third promoter of the IGF-II gene it was possible to delimit a segment of approximately 240bp (pP3MM, containing nucleotides −162 to +74 with respect to exon 3; Figure 3a) which was still capable of responding to co-electroporated c-*jun* and c-*fos* expression constructs (Figure 3b). However, no canonical AP-1 sites were observed in this stretch of sequence.

AP-1 binding sites within *Igf2* P3

The transactivation assays identified a 240bp responsive region in P3 (pBstP3). DNAse I footprinting of this region was carried out using human recombinant c-*jun* protein (Figure 4). Two non-consensus binding sites were identified in the proximal P3 promoter region (site 2, at position −92: GGAGTGGTCAGC and site 1, at position −39: GAGCCACTCAGA, underlining indicates regions of homology to the canonical AP-1 recognition site), as shown in Figure 4b. As a control, footprinting was carried out on the SV40 early promoter-enhancer to demonstrate specific binding to a previously characterised AP-1 site (TTAGTCA, 34) with these experimental conditions (Figure 4c). Footprinting carried out on the pBstP3 template at increasing KCl concentrations indicates AP-1 site 2 to be a higher affinity JUN binding site than site 1 (data not shown).

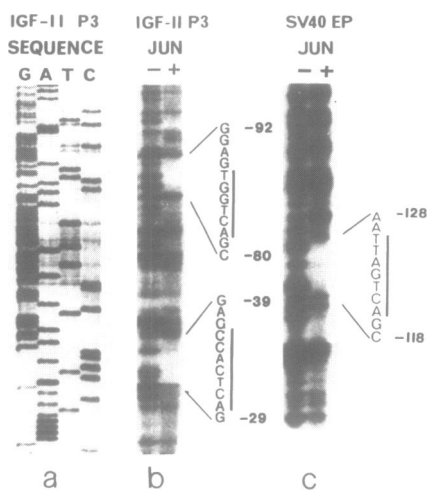

Figure 4. AP-1 binding sites in the proximal promoter region of *Igf2* P3. **a.** Sequence from pBstP3 for direct identification of footprinted sequences. **b.** Pattern of DNAseI degradation of *XhoI*−*KpnI* linearised pBstP3 (coding strand end-labelled at the XhoI site) in the absence (−) or presence (+) of recombinant c-*jun* protein. Sequence of footprinted regions is given (site 1, nucleotide positions −39 to −29; site 2, nucleotide positions −92 to −80) and homology with the AP-1 binding site consensus are indicated. **c.** The SV40 early promoter-enhancer region was employed as a positive control for the footprinting reaction conditions. A previously characterised AP-1 site (34) is indicated.

Functional analysis of the AP-1 sites in *Igf2* promoter 3

The functional importance of the AP-1 binding sites identified in *Igf2* P3 was analysed following polymerase chain reaction (PCR) mediated mutagenesis (Figure 5a). Derivatives of pBstP3 were created containing base substitutions in the AP-1 binding site at position −39 (site 1: CCACTCA in pBstP3 mutated to CCCGGG in pPCR1), in the AP-1 site at position −92 (site 2: TGGTCA in pBstP3 mutated to AGGCCT in pPCR2) and in both sites (sites 1 and 2 mutated in pPCR3). Loss of AP-1 binding to the mutated sites in pPCR1, pPCR2 and pPCR3 was confirmed by gelshift and footprinting analyses (data not shown).

The functional importance of the footprinted sites in *Igf2* P3 was demonstrated by co-electroporation of c-*jun* and c-*fos* expression vectors in transactivation assays utilising the wild type (pWTluc) and mutated (pPCR1luc, pPCR2luc and pPCR3luc)

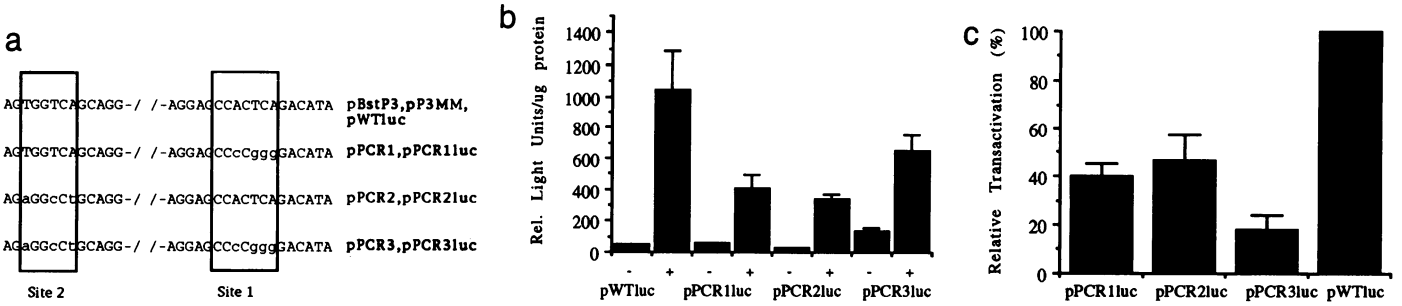

Figure 5. Effects of mutations introduced within the AP-1 sites of *Igf2* P3. **a.** Sequence comparison of constructs with wild type P3 sequences (pBstP3 and pP3MM) with constructs bearing mutations within site 1 (pPCR1, pPCR1luc), site 2 (pPCR2, pPCR2luc), and within both sites (pPCR3, pPCR3luc). **b.** Functional analysis of *Igf2* P3 AP-1 mutants in the presence of c-*jun* and c-*fos* expression vectors in CP-1 ES cells. The results from one representative experiment are given. **c.** The AP-1 dependent transactivation of each mutated version of the P3-luciferase constructs (pPCR1luc, pPCR2luc, and pPCR3luc) expressed as a percentage of the fold transactivation value obtained with the wild type promoter construct (pP3MM). The means and standard errors of three independent experiments are given.

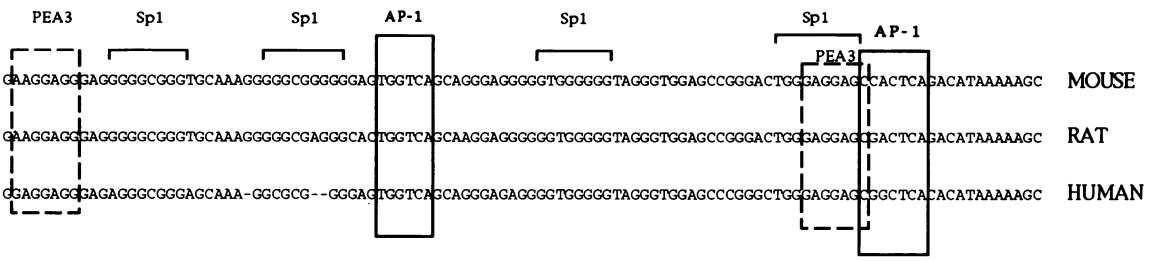

Figure 6. Nucleotide sequence comparison of the *Igf2* P3 region from the mouse (A; ref. 23), rat (B; ref. 6) and human (C; ref. 48) genes. The AP-1 sites characterised in this paper are shown (boxed, solid line), together with Sp1 binding sites mapped in the rat promoter (bracketed; ref. 10) and regions of homology to the PEA3 binding site consensus (NAGGAAG/A, ref. 37; boxed, broken line).

versions of promoter 3-luciferase reporter constructs (Figures 5b and 5c). Mutation of individual sites resulted in approximately 50% reduction of AP-1-mediated transactivation, with the largest effect (an 80% reduction) being observed with the pPCR3luc construct, which lacks both sites.

DISCUSSION

By a combination of functional studies and biochemical techniques we have demonstrated a direct interaction between the AP-1 complex and promoter 3 of the mouse insulin-like growth factor-II gene. This interaction is mediated through two non-consensus AP-1 binding sites (site 1; core CCACTCA, at position −39 to −29, and site 2; core TGGTCA, at position −92 to −80) in the proximal promoter region and can result in a strong activation of the *Igf2* promoter. Mutation of these sites can produce a decrease in the basal activity of the promoter and results in an almost complete elimination of the response to AP-1.

The AP-1 sites (1 and 2) within *Igf2* P3 exhibit substantial divergence from the consensus AP-1 binding site (TGAG/CTC-A; 36, 37); other examples of non-consensus AP-1 sites are however present in the literature (some of which are given in Table 1). Only site 2 is fully conserved between rat, mouse and human *Igf2* P3 sequences (Figure 6) and it is interesting to note that significant differences in relative promoter strength exist between the rodent and human promoters. P4 of the human gene (equivalent to the rodent P3 promoter) appears to be a minor promoter as judged by functional studies and by Northern analysis

Table 1. Comparison of sites protected by c-*jun* in the *Igf2* promoter 3 with characterised AP-1 binding sites and with the consensus AP-1 site

CONSENSUS:	TGANTCA	(33)
COLLAGENASE:	TGAGTCA	(44)
TGFβ-1:	TGAGACA	(45)
SV40:	TTACTCA	(34)
α-FETOPROTEIN:	TGAACATAA	(46)
PROLIFERIN:	TGAGTAG	
	TGATTTG	(47)
c-*myc*	TGATTTA	(49)
Skeletal α-actin	TGGCTCG	(50)
Ovalbumin	TGGGTCA	(51)
c-*jun*	TGACATCA	(52)
Osteocalcin	TTAGTCC	(53)
Igf2 P3: site 1	CCACTCA	
site 2	TGGTCA	

(9), while in rodents P3 seems to be a major contributor to the *Igf2* transcript family (4) and the strongest promoter in the mouse gene as judged by transient transfection studies (Caricasole and Ward, unpublished data). Such species-specific differences in relative P3 promoter strength could be accounted for, at least in part, by qualitative or quantitative variations in transcription factor binding to the P3 promoter region. In this respect it is noteworthy that the human equivalent of mouse AP-1 site 1 deviates considerably from its rodent counterparts and from the AP-1 consensus binding site. Similarly, van Dijk et al (9) failed

to observe protection on the human P3 promoter of sequences bound by Sp1 in the rat P3 region (14), despite extensive sequence homology (Figure 6), and the employment of nuclear extracts from the same cell line (HeLa cells) for footprinting reactions.

The non-synergistic nature of the action of the two *Igf2* P3 AP-1 sites (Figures 5b and 5c) is consistent with the study of Angel *et al* (33) in which multiple TRE elements were introduced into a heterologous promoter. In at least one other case, that of the tissue plasminogen activator type 2 gene (PAI-2), the presence of two AP-1 binding sites in the promoter region has been shown to be necessary for both basal and phorbol ester-inducible transcriptional activity (38). Studies in BRL cells using *Igf2* P3 constructs, either with intact (pWTluc) or mutated AP-1 sites (pPCR3luc) suggest these sites are necessary for full promoter activity (data not shown), drawing a parallel with the PAI-2 gene promoter. Examination of the *Igf2* P3 sequence suggests further similarities with the promoters of other AP-1 responsive genes (Figure 6), in particular the presence of motifs resembling binding sites for the PEA3 transcription factor (consensus AGGAAG/A; 37,44).

In the developing rodent embryo, significant overlaps in the domains of expression of members of the jun and fos gene families and of the IGF-II gene exist (39, 40, 41), in particular in skeletal muscle and developing bone and cartilage, which is consistent with a role for AP-1 in the regulation of IGF-II expression in at least some tissues of the embryo. In the rat, the developmental pattern of *Igf2* mRNA distribution (7, 41) closely resembles that of another AP-1 responsive growth factor gene, namely that encoding TGFβ-1 (42).

The interaction between the AP-1 complex and *Igf2* promoter 3 may be of importance in the observed increase of steady-state *Igf2* message upon ES and EC cell differentiation (18). Levels of c-*jun* message and AP-1 binding activity rise during both EC and ES cell differentiation (16, 17 and this paper), suggesting a role for AP-1 in differentiation-induced gene expression. However, when we introduced a range of *Igf2* promoter-luciferase constructs (including P3-luciferase plasmids) transiently into ES cells cultured in the presence or absence of BRL conditioned medium the differentiation-dependent induction of endogenous *Igf2* expression (Figure 2) was not reproduced by the transfected constructs (data not shown), suggesting that, at least in a transient expression system, the AP-1 complex alone cannot mediate this effect. We are currently engaged in producing stably transformed ES cell lines with a variety of *Igf2* promoter-reporter constructs to test the role of the AP-1 sites during ES cell differentiation. The study of differentiation-inducible gene expression in EC and ES cells has, in at least one case (that of the c-*jun* promoter; ref. 43), shown that promoter constructs transiently introduced into cells failed to produce an induction that could account for the surge in mRNA levels observed for the endogenous gene; the same constructs, when stably transformed into cells, exhibited a strong (up to 180-fold) differentiation-dependent induction of promoter activity.

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