Dual role for transcription factor AP-2 in the regulation of the major fetal promoter P3 of the gene for human insulin-like growth factor II

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The human insulin-like growth factor II (IGF-II) gene contains four promoters that are differentially active during cell growth and development. Promoter 3 (P3) is the most active promoter in fetal and non-hepatic adult tissues. In addition to its expression during development, P3 is also the major promoter in many tumour tissues and IGF-II-expressing cell lines. Here we show that AP-2 has a dual function in P3 regulation *in vivo* as well as *in vitro*. In cells expressing low levels of endogenous AP-2, AP-2 overexpression activates P3, whereas P3 promoter activity is inhibited in cells containing abundant AP-2. Four potential AP-2-binding sites were identified in footprinting studies with recombinant AP-2. One of these AP-2-binding sites is located within the previously identified element P3-4 that contains two adjacent binding sites for IGF-II promoter-binding proteins IPBP3 and IPBP4/5. By applying binding competition assays and mutational analysis it is shown that AP-2 interferes with IPBP3 binding and transactivation *in vivo* as well as *in vitro*. Furthermore, AP-2 can bind additional elements in the proximal P3 promoter that also contribute to AP-2-mediated transactivation as shown by transient transfection assays. From these results we conclude that AP-2 is an important regulator *in vivo* and *in vitro* of IGF-II P3 activity.

Key words: footprinting, gene expression, IGF-II promoter-binding proteins, transcription regulation.

INTRODUCTION

The human insulin-like growth factor II (IGF-II) gene spans 30 kb of chromosomal DNA and is located on chromosome 11 [1]. It consists of nine exons, of which exons 7, 8 and the first part of exon 9 encode the IGF-II prepro-protein. Exons 1, 4, 5 and 6 are preceded by separate promoters (P1 to P4), giving rise to a family of mRNA species of different lengths. Expression of these promoters is regulated in a tissue- and development-specific manner. P1 is exclusively active in adult liver, whereas P2, P3 and P4 are active in most fetal tissues (reviewed in [2]).

Transcription from promoter P3, the major fetal promoter of the IGF-II gene, gives rise to mRNA species of 6.0 and 2.2 kb, depending on the poly(A) signal used. In addition to its activity in fetal tissues, P3 is also up-regulated in many human tumours of various origins and in many tumour-derived cell lines [3,4]. P3 spans approx. 1300 nt upstream of the transcription start site of exon 5 and consists of 70–80% GC base pairs. Transient transfection assays have revealed that the region between −289 and the transcription start site (+1) supports basal activity in HeLa and Hep3B cells [5,6]. Four elements were identified in this proximal promoter region that were bound by nuclear factors from HeLa cells in footprint analysis. P3-1 (−90 to −63) contains an inverted CCAAT box; P3-2 (−131 to −103) encompasses an Sp1 binding site. P3-3 (−146 to −128) binds an unknown protein; P3-4 (−193 to −172) is composed of two protein-binding sites, box A (−193 to −188) bound by IGF-II promoter-binding protein (IPBP)4/5, and box B (−183 to −172) bound by IPBP3 [6,7]. Element P3-4 also contains a consensus sequence for AP-2; here we examine the putative binding of AP-2 to the IGF-II promoter P3 and its function in transcription of P3.

AP-2 binds to DNA as a dimer and recognizes the palindromic sequence element 5′-GCCNNNGGC-3′ [8]. This 50 kDa transcription factor was initially identified as a regulator of human metallothionein IIa expression [9,10]. AP-2 has been found to be involved in the regulation of many viral and cellular genes, such as SV40 [10], proenkephalin [11], IGFBP-5 [12], HTLV-I [13] and VGF/VEGF [14]. In addition to its gene activation potential, AP-2 can also inhibit the transcription of a number of genes. C/EBPα gene expression is repressed by AP-2 during adipogenesis [15]. AP-2 has also been shown to function as a repressor for a number of other genes, such as K3 keratin [16], prothymosin [17] and acetylcholinesterase [18].

Here we examine the role of AP-2 in the regulation of the human IGF-II gene promoter P3. We find that the proximal region of P3 up to position −289 contains four AP-2-binding sites. We also show that AP-2 is able to activate P3 in a dual fashion: expression of AP-2 leads to the activation of gene activity in cells expressing small amounts of AP-2, whereas overexpression of AP-2 leads to the inhibition of P3 activity. Furthermore, we show that AP-2 competes for binding with IPBP3, a protein important for the activity of human IGF-II.

MATERIALS AND METHODS

Oligonucleotides and plasmids

The following oligonucleotides were provided by Pharmacia (Uppsala, Sweden): M3upper, 5′-CCGGG**TAAT**CAGGGCCC-CCAGCCCGCACCCCCCGCCCGCTCTTGGC-3′; M3lower, 5′-CCGAGCCAAGAGCGGGGCGGGGGTGCGGGCTG-GGGGCCCT**GATTA**C-3′. Other oligonucleotides used, P3-4, P3-4M1 and P3-4M3, were as described previously [6]. The AP-2 consensus oligonucleotide pair 5′-GATCGAACTGAC-CGCCCGCGGCCCGT-3′ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The IGF-II promoter P3 expression construct −289P3 (pFlag-*Xba*) and the adenovirus

Abbreviations used: AdML, adenovirus major late; EMSA, electrophoretic mobility-shift assay; fpu, footprinting unit; IGF, insulin-like growth factor; IPBP, IGF-II promoter-binding protein; rhAP-2, recombinant human AP-2; WCE, whole-cell extract.

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major late promoter template (pAdML200) were as described previously [6]. pFlag constructs contain both a G-less cassette, to monitor *in vitro* transcription rates, and a luciferase reporter gene, to monitor promoter activity in transient transfection assays. −289P3M3 (pFlag-*Xba*M3), a mutant at positions −180 to −177, was created by replacing an *Ava*1 fragment containing box B with the double-stranded oligonucleotide M3upper/lower. All constructs were checked by nucleotide sequence analysis. CMV-AP2 was kindly provided by Dr. M. Eilers (Marburg, Germany).

Cell culture and transient transfections

Hep3B cells were cultured in α-modified minimal essential medium; HeLa cells were cultured in Dulbecco's modified Eagle's medium. Media were supplemented with 10% (v/v) fetal calf serum, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 300 μg/ml glutamine. For transfection, HeLa and Hep3B cells were seeded at a density of 3×10^5 cells per 3 cm dish. Fresh medium was added 2 h before transfection. Introduction of expression constructs into Hep3B and HeLa cells was performed by means of a modified version of the calcium phosphate co-precipitation method [19]. In a standard transfection, 2 μg of reporter construct was co-transfected with 2 μg of expression plasmid. As an internal control, 0.25 μg of RSV-LacZ was added to the transfection mixture. Cells were exposed to the DNA/calcium co-precipitate for four hours, then fresh medium was added and after 24 h the cells were harvested for the determination of luciferase and β-galactosidase activities. Preparations of cell extracts for the β-galactosidase assays and the luciferase assays were performed by using standard procedures as described [19,20].

Preparation and fractionation of cell extracts

For the preparation of whole-cell extract (WCE), HeLa cells were grown in suspension cultures to a density of 5×10^5 cells/ml. Extract preparation was performed by the method of Manley et al. [21]. The protein concentrations were determined by the Bradford protein quantification assay (Bio-Rad, Brussels, Belgium). WCE was prepared from 20 g of human HeLa cells (400 mg of protein in 100 mM KCl). For the fractionation of HeLa WCE the extract was applied to a DEAE-Sepharose column that was eluted with 100 and 300 mM KCl. The 100 mM KCl fractions containing IPBP4/5 were pooled and subsequently applied to a heparin–Sepharose column that was eluted with a linear gradient from 100 to 1000 mM KCl. The 300 mM KCl fractions from the DEAE column containing IPBP3 were applied to an SP-Sepharose column and eluted with a linear gradient of 100–1000 mM KCl. Fractions containing either IPBP3 or IPBP4/5 were dialysed extensively against 100 mM KCl.

Electrophoretic mobility-shift analysis (EMSA)

Double-stranded oligonucleotides were end-labelled by phosphorylation with [γ-^{32}P]ATP and T4-kinase [19]. EMSA binding reactions were performed as described previously [22]. In brief, binding reactions were performed by mixing DNA probes, double-stranded competitor DNA and binding buffer [20 mM Hepes/KOH (pH 7.6)/1 mM $MgCl_2$/75 mM KCl/1 mM dithiothreitol/0.018% (v/v) Nonidet P40, containing 1 μg of poly(dI-dC) (Pharmacia, Uppsala, Sweden)], after which protein extract was added. The total reaction volume of 15 μl was incubated for 60 min on ice. Bound and unbound probes were separated on a 5% (w/v) polyacrylamide gel (37.5:1) in 0.5 × Tris/borate/EDTA. After the gel had been dried, the DNA–protein complexes were detected by autoradiography. Purified recombinant human AP-2α (rhAP-2α) was obtained from Promega (Madison, WI, U.S.A.) and supplied as a solution of 1 footprinting unit (fpu)/μl. The antibodies against AP-2α and CDK-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). EMSA binding competition experiments were performed by preincubation of a constant amount of IPBP3 fraction (2 μl) with P3-4 for 30 min, after which increasing amounts of rhAP-2 (0.2 and 0.4 fpu) were added. In the converse experiment, a constant amount of rhAP-2 (0.4 fpu) was pre-incubated for 30 min with P3-4, after which increasing amounts IPBP3 (1 and 2 μl) were added.

DNase I footprinting

DNase I footprinting reactions were performed as described previously [22]. The probe for footprinting was an *Eco*RI/*Sac*I fragment containing the promoter region −290 to −7 labelled in the lower strand. This probe was incubated with 4 fpu of AP-2, after which 0.01 or 0.02 unit of DNase I was added for 90 s. After the reaction had been stopped by extraction with phenol and precipitation with ethanol, the mixtures were applied to a 10% polyacrylamide/urea gel. After electrophoresis the gel was dried and exposed to Fuji X-ray film.

Transcription reactions *in vitro*

Transcription reactions *in vitro* were performed as described previously [6]. Plasmid DNA species were purified twice on CsCl gradients and subsequently dialysed extensively against 10 mM Tris/HCl/1 mM EDTA (pH 8.0). A typical transcription reaction *in vitro* contained 100 ng of −289P3 or −289P3M3 template and 200 ng of pAdML200 as an internal control. The amount of WCE used in each reaction was 75 μg. For antibody experiments, 1, 2 or 3 μl of the antibody solution was pre-incubated for 15 min with the WCE, after which the transcription reactions were performed as described above.

RESULTS

IGF-II P3-4 promoter element contains an AP-2-binding site

In previous experiments we identified an essential element for the basal activity of the human IGF-II promoter P3, designated P3-4 (Figure 1a). This element was shown to bind five proteins present in HeLa and Hep3B cell extracts (Figure 1b) [6]. Protein 2 was shown to be a non-specific binding protein. Protein 3 (IPBP3) and proteins 4/5 (IPBP4/5) are specific and present in comparable amounts in these two cell lines, whereas the amount of protein 1, IPBP1, depended on the cell type and was expressed at much higher levels in HeLa cells than in Hep3B cells. Two binding sites within this element have been identified, box A (−193 to −188), which is bound by IPBP4/5, and box B (−183 to −172), which is bound by IPBP3 (Figure 1a).

In addition to these two boxes this element also contains a near-consensus AP-2-binding site 5′-CCCCGGGGC-3′ (−187 to −179). To determine whether one of the P3-4-binding complexes from HeLa cells contains AP-2, a supershift experiment was performed with antibody against AP-2. Figure 1(b) shows that the antibody against AP-2 supershifted IPBP1, whereas IPBP3 and IPBP4/5 binding is not affected, strongly indicating that IPBP1 could be AP-2. Conversely, it was shown that when HeLa cell extract was incubated with the AP-2 consensus oligonucleotide probe, a complex was formed with the same mobility as IPBP1 which bound the P3-4 oligonucleotide probe. Furthermore, recombinant human AP-2 bound to both

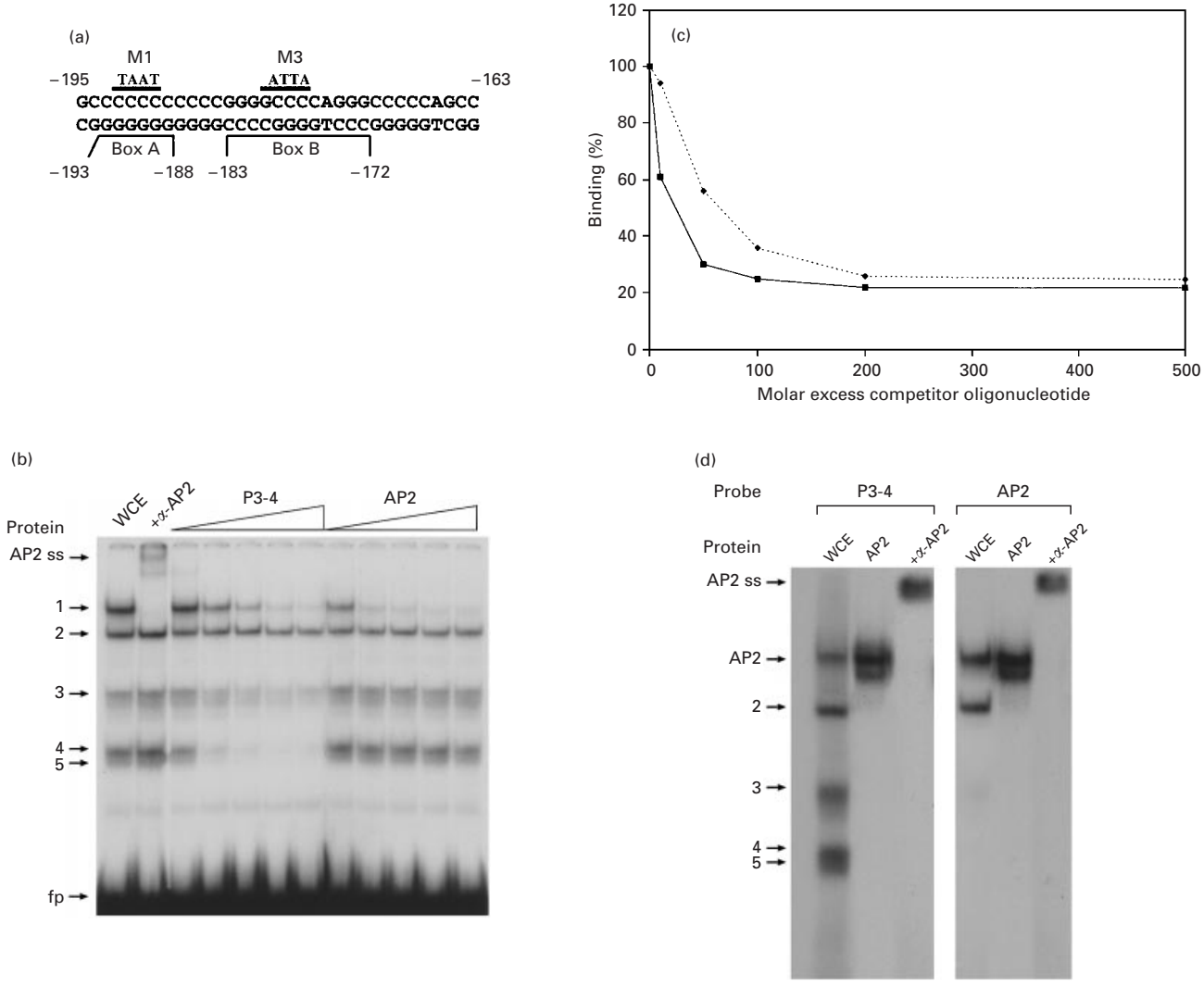

Figure 1 Transcription factor AP-2 binds to element P3-4

(**a**) Schematic overview of the different protein-binding regions of the P3-4 element. Mutations M1 and M3 used in the EMSA experiments are indicated. Box A (−193 to −188) binds IPBP4/5, and box B (−183 to −172) binds IPBP3. (**b**) EMSA experiment with 2 μg of HeLa WCE and with P3-4 as probe; the five protein–DNA complexes IPBP1 to IPBP5 are indicated at the left (1 to 5). In lane 2, 1 μl of antibody against AP-2 (α-AP2) was added, resulting in an AP-2 supershift (AP-2 ss). The remaining two groups of five lanes represent EMSA-competition experiments with 10-fold, 50-fold, 100-fold, 200-fold and 500-fold molar excesses of competitor oligonucleotides P3-4 and AP-2 respectively. Abbreviation: fp, free probe. (**c**) Representation of the EMSA competition experiment from (**b**). The percentage binding of AP-2 is plotted against the molar excess of competitor oligonucleotide P3-4 (dotted line) or AP-2 (solid line) that was added. Calculations were done after quantification on a PhosphorImager; 50% binding was observed at 25-fold molar excess for AP-2 and 75-fold molar excess for P3-4. (**d**) Recombinant human AP-2 binds P3-4. In the left panel, P3-4 oligonucleotide was used as a probe; in the right panel, AP-2 oligonucleotide was used as probe. HeLa WCE (2 μg) and rhAP-2α (0.2 fpu) were used in this EMSA experiment. In lanes 3 and 6, 1 μl of anti-(AP-2) antibody (α-AP2) was preincubated with 0.2 fpu of rhAP-2. Protein–DNA complexes IPBP2 to IPBP5 (2 to 5), AP-2 and the AP-2 supershift (AP-2 ss) are indicated at the left. Note that the binding of recombinant human AP-2 gives rise to the formation of two AP-2 complexes [32]. Because both complexes are supershifted by AP-2 antibody we assume that the second complex is generated by truncated AP-2 generated by alternative translational start or stop signals.

the P3-4 and the AP-2 consensus oligonucleotide probes and co-migrated with IPBP1 from HeLa WCE (Figure 1d). After the addition of AP-2 antibody, the AP-2 complexes were supershifted (Figure 1d).

Subsequently, in an EMSA competition experiment, the consensus AP-2 oligonucleotide competed efficiently only with IPBP1, whereas the P3-4 oligonucleotide competed with all specific P3-4-binding complexes (Figure 1b). The fact that 3-fold more P3-4 competitor was needed than AP-2 competitor to decrease the binding of complex 1 to 50% indicated that the affinity of AP-2 for element P3-4 was one-third of that for the AP-2 consensus oligonucleotide (Figure 1c). From these results we conclude that AP-2 can bind to element P3-4 of the IGF-II promoter P3, resulting in formation of the previously unidentified complex IPBP1.

AP-2 and IPBP3 compete for binding to element P3-4

To study the competition of IPBP3, IPBP4/5 and AP-2 binding to element P3-4, partly purified protein fractions containing IPBP3 or IPBP4/5, or recombinant AP-2, were used for DNA binding studies with P3-4 and P3-4 mutant oligonucleotides as probes (Figure 2, upper panel). As shown previously, box A (−193 to −188) is bound by IPBP4/5 and box B (−183 to

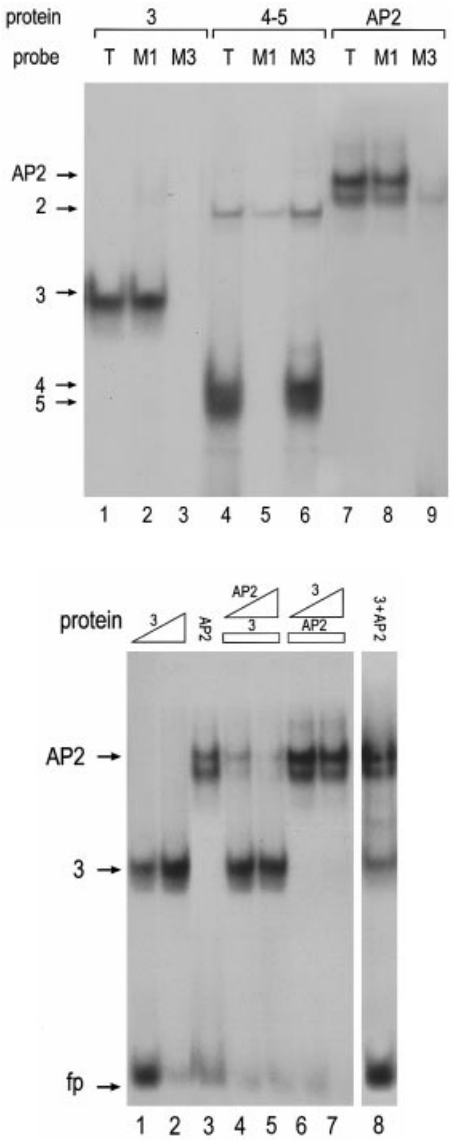

Figure 2 AP-2 and IPBP3 have overlapping binding sites on the P3-4 element

Upper panel: binding of IPBP3, IPBP4/5 and AP-2 to different P3-4 mutants in an EMSA experiment. Different protein fractions enriched for IPBP3 (2 μl), IPBP4/5 (2 μl) or AP-2 (0.2 fpu) were bound to double-stranded oligonucleotides representing wild-type P3-4 (T), P3-4M1 (M1) or P3-4M3 (M3). Lower panel: AP-2 competes with IPBP3 for P3-4 binding. EMSA experiment in which 1 or 2 μl of IPBP3-enriched fraction (lanes 1 and 2) or 0.2 fpu of rhAP-2 (lane 3) was incubated with the P3-4 probe. In lanes 4 and 5, 2 μl of IPBP3-enriched fraction was preincubated for 30 min with P3-4, after which increasing amounts of rhAP-2 (0.2 fpu in lane 4 and 0.4 fpu in lane 5) were added. Conversely, 0.2 fpu of rhAP-2 was preincubated with the probe, after which 1 or 2 μl of IPBP3 was added (lanes 6 and 7). Lane 8 shows the binding of 2 μl of IPBP3 fraction and 0.4 fpu of AP-2 when added simultaneously to the probe. Abbreviation: fp, free probe. The significance of the labels at the left is the same as in Figure 1(**d**).

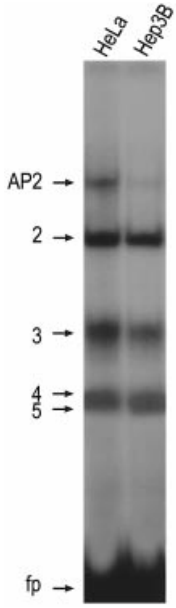

Figure 3 AP-2 is expressed at much lower levels in Hep3B cells than in HeLa cells

EMSA experiment with 2 μg of HeLa and Hep3B WCE and with double-stranded P3-4 as probe. The significance of the labels at the left is the same as in Figure 1(**d**). Abbreviation: fp, free probe.

-172) is bound by IPBP3 [6]. The introduction of mutations into box A (M1 mutant oligo) disrupted IPBP4/5 binding but had no effect on IPBP3 binding or AP-2 binding. The introduction of mutation M3 in box B disrupted IPBP3 binding and also disrupted binding of AP-2 to the oligonucleotide (Figure 2, upper panel). These results indicate that IPBP3 and AP-2 share a common binding region in box B of the P3-4 element and that AP-2 does not bind to box A.

Since AP-2 and IPBP3 can both bind to box B of P3-4, we investigated binding of AP-2 and IPBP3 to P3-4 in more detail. In order to see if AP-2 could interfere with IPBP3 binding, we preincubated oligonucleotide probe P3-4 with IPBP3 and subsequently added recombinant AP-2 in increasing amounts (Figure 2, lower panel). This showed that the preincubation of IPBP3 with P3-4 inhibits subsequent binding of AP-2. Conversely, when the preincubation was done with recombinant AP-2 protein followed by IPBP3 addition, AP-2 inhibited the binding of IPBP3 to P3-4 (Figure 2, lower panel). Furthermore, when endogenous AP-2 was bound to element P3-4, complex IPBP1 could also be competed away when increasing amounts of partly purified protein 3 were added (results not shown). From these experiments we conclude that IPBP3 and AP-2 can bind element P3-4 in a mutually exclusive manner.

Regulation of IGF-II P3 activity by AP-2 *in vivo*

Because both AP-2 and IPBP3 can bind to the same element in P3, the concentration ratio of both proteins in a particular cell type might determine the effect on transcription. In fact, AP-2 has been described as a potent transactivator of several promoters [10,12,13]. In addition, it has become clear that the effect of enhanced AP-2 expression can result in the repression of transcription, depending on the basal AP-2 level in various cell types [12,23,24].

To test whether this dual effect could also be observed for the IGF-II gene, we selected two different cell lines in which basal AP-2 levels differed drastically, HeLa and Hep3B. To compare the endogenous AP-2 levels in HeLa and Hep3B cells, an EMSA experiment was performed with element P3-4 and WCE of both cell lines (Figure 3). HeLa cells contain abundant AP-2 protein,

whereas the expression of AP-2 in Hep3B cells is low and the AP-2 complex is hard to detect under similar conditions. Furthermore, the expression levels of IPBP3 and IPBP4/5 were similar in both cell lines (Figure 3). Using these two cell lines in transient transfection experiments in which 2 μg of a P3(−289 to −7)–luciferase construct, 2 μg of CMV-AP2 and 0.25 μg of CMV-lacZ were co-transfected, we determined the activation of promoter P3 by AP-2 in HeLa and Hep3B cells. In Hep3B cells, co-transfection of AP-2 and P3(−289 to −7) resulted in a 3.8-fold activation of the IGF-II P3 promoter, whereas in HeLa cells the co-transfection of similar amounts of AP-2 expression plasmid inhibited P3 promoter activity by 50%. These results were confirmed for two additional cell lines. In 293 cells, which contain endogenous AP-2 levels comparable to those in HeLa cells (results not shown), P3 activity was also inhibited by 50% when AP-2 was co-transfected. In contrast, in AP-2-deficient HepG2 cells [8] AP-2 could efficiently activate IGF-II P3 again as in Hep3B cells (results not shown). These results suggest that the overexpression of AP-2 in cells already endogenously expressing AP-2 (HeLa and 293) results in an inhibition of promoter activity, whereas in cells expressing very small amounts of AP-2, or none (Hep3B and HepG2), promoter P3 can be activated 3–4-fold by co-transfected AP-2.

Regulation of IGF-II P3 activity *in vitro* by AP-2

To support the results obtained with the transient co-transfection experiments, the regulation of P3 by AP-2 was also studied *in vitro*. Transcription reactions *in vitro* were performed with −289P3 and HeLa extract and this basal activity was set at 100%. To determine whether the endogenously expressed AP-2 in HeLa cells was sufficient to activate promoter P3 fully, similar transcriptions *in vitro* were performed after preincubation of HeLa WCE with increasing amounts of antibody against AP-2 (Figure 4, upper panel). The addition of specific anti-(AP-2) to the WCE before transcription from the −289P3 template inhibited transcription in a dose-dependent manner, showing that endogenous AP-2 in HeLa cells transactivates P3 *in vitro*. Transcription from the adenovirus major late promoter (AdML) construct that served as an internal control was not affected by the addition of anti-(AP-2). Also, the addition of a non-specific anti-CDK2 antibody to the transcription reactions had no effect on the transcription of either the IGF-II −289P3 template or the AdML template (Figure 4, upper panel).

To investigate whether the overexpression of AP-2 *in vitro* was correlated with the data *in vivo* of transcriptional inhibition, we performed transcription reactions *in vitro* with HeLa WCE in the presence of increasing amounts of rhAP-2. Figure 4 (lower panel) shows that the addition of recombinant AP-2 to the HeLa WCE resulted in a dose-dependent inhibition of P3 activity, whereas expression from the AdML template remained unaffected. Also, the addition of the non-specific protein BSA at similar concentrations had no significant effect on the P3 activity levels (Figure 4, lower panel).

Interestingly, these results show that HeLa cells contain sufficient AP-2 to activate P3 *in vitro* and show that increasing the endogenous AP-2 level by adding recombinant AP-2 results in an inhibition of P3 activity. These results correlate well with the results obtained *in vivo* showing that too much AP-2 leads to inhibition of P3 activity.

Effect of box B disruption on transactivation by IPBP3 and AP-2 *in vitro*

Both AP-2 and IPBP3 can bind to element P3-4, and the introduction of the M3 mutation disrupts the binding of the two

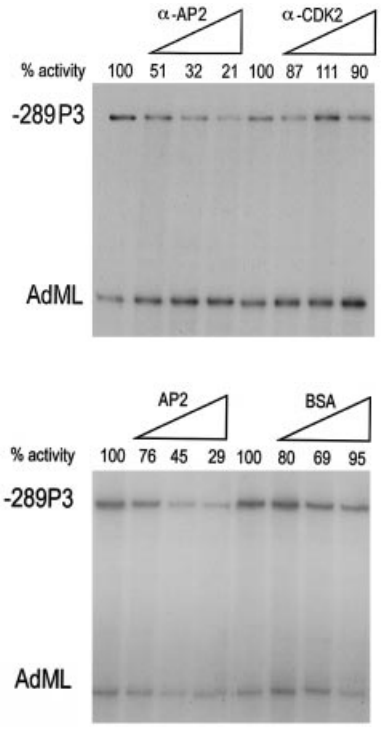

Figure 4 AP-2 regulates P3 activity *in vitro*

Upper panel: transcription *in vitro* from −289P3 with HeLa WCE is inhibited by antibodies against AP-2. Transcription reactions *in vitro* were performed with the −289P3 template in the presence of increasing amounts (1, 2 and 3 μl) of anti-(AP-2) (α-AP2) or anti-CDK2 (α-CDK2). Lower panel: the addition of rhAP-2 (AP2) to HeLa WCE inhibits transcription *in vitro* from −289P3. Increasing amounts of rhAP-2 (1, 2 and 3 fpu; left four lanes) or similar amounts of BSA (right four lanes) were added to the transcription reactions. The AdML promoter was included in all transcription reactions as an internal standard for transcription. Percentage activity represents the P3 signal after normalization to the AdML signal. Numbers indicate the percentage of transcription activity of −289P3 compared with its basal transcription, which was arbitrarily set at 100%.

proteins. To investigate the effect of each protein on P3 activity, we introduced the M3 mutation into the −289P3 reporter construct. Using limiting amounts of HeLa WCE for transcription assays *in vitro*, we examined the effects of the addition of IPBP3 (Figure 5, upper panel) or the addition of antibody against AP-2 (Figure 5, lower panel). The activity of the wild-type constructs could be enhanced by up to 2.8-fold when the transcription mixture was supplemented with additional IPBP3 protein, whereas AdML transcription was not affected. The addition of IPBP3 to the −289P3M3 mutant template did not lead to enhanced transcription, suggesting that IPBP3 stimulation occurs through binding to the P3-4 element only (Figure 5, upper panel).

The effect of the M3 mutation on transactivation by AP-2 was investigated by the addition of increasing amounts of antibody against AP-2 to the WCE by using the −289P3 and −289P3M3 templates (Figure 5, lower panel). Transcription from the wild-type −289P3 as well as the mutant −289P3M3 template was inhibited with similar kinetics after the addition of antibody against AP-2 to the transcription assays *in vitro*. From this we conclude that AP-2 is an activator of P3 transcription, but that in addition to the AP-2-binding site in P3-4, additional AP-2-binding sites might be present in this −289P3 promoter construct that contribute to AP-2 activated transcription.

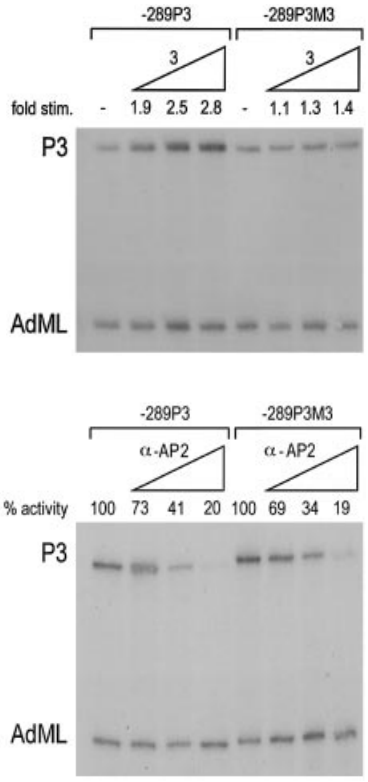

Figure 5 Mutation of element P3-4 abolishes activation by IPBP3 but not by AP-2

Upper panel: activation of transcription *in vitro* by IPBP3 is abolished by the introduction of M3. Transcription reactions *in vitro* with HeLa WCE were performed with −289P3 and −289P3M3 in the presence of 3, 6 or 9 μl of an IPBP3-enriched protein fraction purified from HeLa cells. Lower panel: the introduction of M3 does not abolish transcriptional activation by AP-2. Transcription reactions *in vitro* were performed with −289P3 and −289P3M3 in the presence of 1, 2 or 3 μl of antibody against AP-2. Percentage activity represents the P3 activity after normalization to the AdML signal. Numbers indicate the percentage of transcription activity of −289P3 and −289P3M3 compared with their basal transcription, which was arbitrarily set at 100%. These experiments were performed in conditions under which the amount of HeLa extract was limiting for the transcription reactions. α-AP2, anti-AP2 antibody.

AP-2 binds four sites in the proximal P3 promoter region

To investigate further the presence of potential AP-2-binding sites within the proximal P3 promoter region, footprinting experiments were performed with purified recombinant AP-2 protein, and the proximal promoter region encompassing the region from positions −290 to −7 relative to the transcription start site was used as a probe (Figure 6, upper panel).

No AP-2-binding sites were present in the region between −290 and −190 and in the region between −190 and −7 four AP-2-binding sites could be identified. The AP-2 footprint found on the P3-4 element (−187 to −173) partly overlaps box B (−183 to −172), and box A (−193 to −188) lies adjacent to this AP-2-binding site (Figure 6, lower panel). This confirms the EMSA results that AP-2 and IPBP3 have overlapping binding sites and can bind mutually exclusive to box B. Of the four AP-2-binding sites, sites 2 and 4 contain a perfect AP-2 consensus binding site (5′-GCCNNNGGC-3′), whereas sites 1 and 3 contain a single mismatch to the consensus sequence (Figure 6, lower panel).

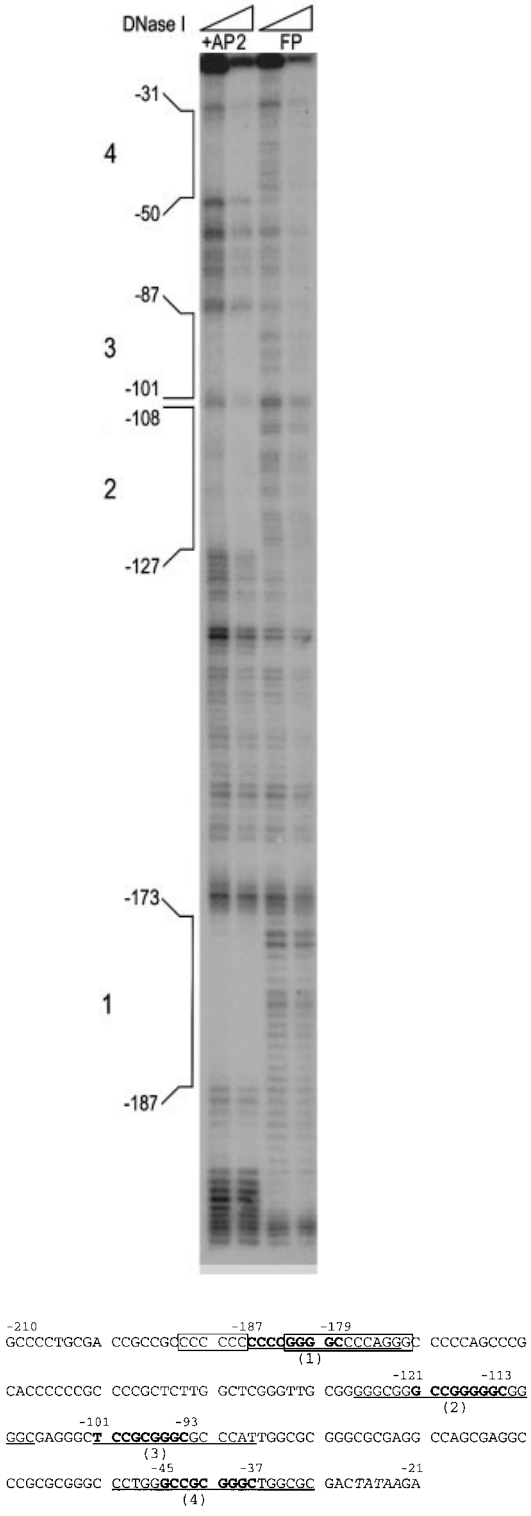

Figure 6 AP-2 binds to multiple sites of the proximal P3 promoter region

Upper panel: DNase I footprinting with rhAP-2 and with the proximal P3 promoter region (−290 to −7) as a probe. Lanes 1 and 2, 4 fpu of recombinant human AP-2α was added with increasing amounts of DNase I; lanes 3 and 4, no protein was added. The regions protected from DNase I cleavage are indicated at the left. Abbreviation: fp, free probe. Lower panel: sequence of the proximal P3 promoter and the P3 regions protected by AP-2 from DNase I cleavage (underlined). The core consensus AP-2 sequence (5′-GCCNNNGGC-3′) is indicated in bold type in all protected regions. The AP-2-binding sites numbered 1–4 are indicated. Box A and box B are indicated by boxes.

AP-2 sites 2, 3 and 4 are functional in mediating transactivation by AP-2 in Hep3B cells

Because the transcription results *in vitro* indicated that, in addition to the P3-4 AP-2-binding site, other AP-2-binding sites are involved in P3 activation as well, co-transfection experiments were performed with the P3M3 mutant and AP-2. Using 2 μg of -289P3M3 mutant reporter construct, 2 μg of CMV-AP2 and 0.25 μg of CMV-LacZ we investigated the effect of AP-2 on P3 activity. As expected, AP-2 still activated transcription 3.6-fold when the P3-4 AP-2-binding site was disrupted. These experiments indicate that the additional AP-2 sites present in the proximal IGF-II promoter are also functional and contribute to the activation of P3 by AP-2.

DISCUSSION

AP-2 is an important regulator of the activity of the IGF-II promoter P3. As was shown by footprinting analysis, purified AP-2 can bind to four different binding sites within the proximal P3 region located from -290 to -7 relative to the transcription start site. However, under less favourable conditions for footprinting, when using WCE from HeLa cells instead of purified AP-2, it was previously shown that not all four potential AP-2 sites are actually protected by proteins. Regions protected by HeLa nuclear extract were previously identified and designated P3-1 (-90 to -63), P3-2 (-131 to -103), P3-3 (-146 to -128) and P3-4 (-192 to -172) [5–7]. By using HeLa cell extract, containing sufficient AP-2, only two of the four potential AP-2-binding sites, AP-2 site 1 and AP-2 site 2, were actually protected by protein, suggesting that in HeLa cells these are the actual AP-2-binding sites under physiological conditions. Of the two additional AP-2-binding sites (3 and 4) identified when purified recombinant AP-2 protein was used, AP-2-binding site 4 is not transcriptionally active because transcription experiments *in vitro* and transient transfection showed that for the -89P3 construct no transcriptional activity can be detected with HeLa cell extracts [5]. AP-2-binding site 3 might become functional at higher AP-2 concentrations.

The disruption of AP-2 site 1 did not affect transcriptional activation by AP-2, suggesting that the inactivation of one AP-2-binding site is not enough to inhibit transcriptional activation by AP-2. This has also been shown for the H^+-ATPase B2 subunit promoter, in which the deletion of seven out of eight AP-2-binding sites still resulted in a good response to exogenously added AP-2, suggesting that a single AP-2 site can account for a large part of the transcriptional response to AP-2 [25]. For the transcriptional activation of the HTLV-I LTR by AP-2 at least two out of three AP-2-binding sites are required [13].

The IGF-II promoter P3 can be activated 3–4-fold by AP-2 in co-transfection experiments of Hep3B and HepG2 cells, which do not express significant amounts of AP-2. In contrast, in co-transfection studies of AP-2 and IGF-II P3 in HeLa and 293 cells, already endogenously expressing AP-2, an inhibition of transcriptional activity was observed. This was confirmed by transcription experiments *in vitro* with HeLa extract, in which additional AP-2 also inhibited the activity of promoter P3, suggesting that AP-2 has a dual role in the control of IGF-II P3 expression. Because Hep3B and HepG2 cells contain hardly any AP-2, whereas 293 and HeLa cells express AP-2 abundantly, the endogenous AP-2 level of a particular cell type determines the effect on P3 activity. A dual role has also been described for the mouse cartilage-derived retinoic acid-sensitive protein gene CD-RAP, in which the enhanced expression of endogenous AP-2 after the treatment of chondrocytes with retinoic acid resulted in an inhibition of CD-RAP promoter activity, whereas the CD-RAP promoter could be efficiently activated by AP-2 in AP-2-deficient HepG2 cells [24]. This dual role for AP-2 has also been described for the human HOX A4 gene promoter and the IGFBP-5 promoter [12,23].

Of several reported mechanisms by which AP-2 can inhibit gene transcription, one is relevant for IGF-II P3 regulation. This mechanism has been named transcriptional 'self-interference'. It is thought that high-level expression of AP-2 results in the squelching of cofactors needed for AP-2 function, resulting in the inhibition of transactivation by AP-2 [26]. Because the inhibition of P3 activity is observed only in AP-2 overexpression in HeLa and 293 cells, this suggests that 'self-interference' might contribute to an inhibition of P3 activity in this case.

P3-4 is an important element for the basal activity of P3 in HeLa and Hep3B cells. In this paper we demonstrate that IPBP3 and AP-2 have partly overlapping binding sites and that the binding of the two proteins is mutually exclusive. Furthermore, we showed in EMSA experiments that AP-2 binding to element P3-4 can interfere with IPBP4/5 binding, most probably caused by steric hindrance of the adjacent binding sites (results not shown).

In view of these results, it is obvious that the individual concentration of the proteins capable of binding element 3-4 eventually determines the effect on IGF-II transcription. Differences in binding affinity and the ratio of IPBP3 and AP-2 determine which protein binds this element preferentially. In cells expressing low levels of AP-2 such as Hep3B cells, the activation of P3 will be mediated by the binding of IPBP3 and IPBP4/5 to P3-4. Indeed it was shown by transcription experiments *in vitro* that IPBP3 can activate P3 by binding to box B, as was shown previously for IPBP4/5 as well [6]. In cells expressing both AP-2 and IPBP3, such as HeLa cells, competition between the two factors for box B determines which protein activates P3.

In conclusion, we identified four AP-2-binding sites in the proximal region of the human IGF-II P3 promoter, and by transcription *in vitro* and by co-transfection experiments we showed that AP-2 can activate this promoter approx. 3–4-fold. Furthermore, we showed that there is a dual role for AP-2 in the regulation of IGF-II P3 expression: overexpression leads to the inhibition of promoter activity, whereas expression at a lower level can transactivate the promoter.

The physiological importance of AP-2 became clear from the targeted disruption of the gene in mice [27]. AP-2-null mice die at birth as a result of severe defects during neural development, such as defects in the formation of cranial ganglia and the failure of neural tube closure. This indicates that AP-2 is an important regulator during fetal growth and differentiation.

From targeted disruption of the IGF-II gene in mice it was concluded that IGF-II is an essential growth factor during fetal development [28]. Interestingly, both AP-2 and IGF-II expression can be up-regulated by retinoic acid during development [10,29–31]. This concerted regulation of AP-2 and IGF-II might provide a clue to the physiological importance of these factors in development.

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