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# The c-*jun* Proto-oncogene Down-regulates the Rat α-Fetoprotein Promoter in HepG2 Hepatoma Cells without Binding to DNA*

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**Brigitte Bois-Joyeux‡, Mikhail Denissenko‡§¶, Hélène Thomassin‡, Sophie Guesdon‡, Raina Ikonomova‡‖, Dominique Bernuau§, Gérard Feldmann§, and Jean-Louis Danan‡****

*From the ‡Centre de Recherche sur l'Endocrinologie Moléculaire et le Développement, CNRS, UPR 1511, F92190, Meudon, France and §INSERM U 327, Structure et Fonctions des Cellules Hépatiques, Faculté de Médecine Xavier Bichat, F75018, Paris, France*

**The effects of a phorbol ester (TPA) and of members of the Jun and Fos oncoprotein family on the activity of the rat α-fetoprotein (AFP) promoter were checked by using transient expression experiments in HepG2 hepatoma cells. TPA blocked the activity of the rat AFP promoter in a dose-dependent manner. Overexpression of c-Jun specifically repressed the rat AFP promoter but not the albumin promoter. JunB and JunD were poorer inhibitors. c-Fos expression did not potentiate the negative effect of Jun. The Jun-induced repression does not require binding of c-Jun to the AFP promoter. DNase 1 footprinting experiments did not display any high affinity binding site for Jun on the AFP promoter. Integrity of the c-Jun DNA binding domain is not required for the c-Jun protein to block the AFP promoter. The N-terminal part of Jun, which contains the activating domain, is responsible for the repression as shown by using Jun-Gal4 chimera. Jun likely exerts its negative control on the AFP promoter via protein-protein interactions with a not yet identified *trans*-activating factor within the −134 to +6 region or with a component of the general machinery of transcription. Jun proteins can thus be key intermediates in regulatory cascades which result in the differential modulation of the AFP and albumin gene expression in the course of liver development and carcinogenesis.**

The α-fetoprotein gene (AFP),[1] which belongs to the same family as the albumin gene, is expressed in a very tightly controlled manner in the course of mammalian development. It is specifically expressed at a high rate in the yolk sac, the fetal liver, and to a lesser extent in the fetal gut. α-Fetoprotein, whose function is still not fully understood, is the most abundant plasma protein during the fetal period. Transcription of the AFP gene drops abruptly around birth and is virtually totally blocked during normal adult life. Expression of the AFP gene can, however, be resumed under certain pathophysiological conditions, such as liver regeneration after partial hepatectomy or chemical injury and in hepatocarcinogenesis (see Ref. 1 for review). The AFP gene, whose expression is controlled by multiple signal transduction pathways, is a powerful model with which to examine the molecular mechanisms that dictate the liver specificity of gene expression and which modulate its activity during development and cancerogenesis.

The AFP gene is mainly regulated at the transcriptional level. It is now clear that the promoter region confers the liver specificity of expression and that upstream positive and negative regulatory elements, which are strongly liver-specific, can also control the transcription rate of the AFP gene (see Refs. 2–4 for reviews). Several of the liver-enriched transcription factors which may participate in the functioning of the AFP promoter and enhancers in the liver cell have been characterized (5–12).

Transfection experiments showed that the activities of the rat AFP promoter and enhancer at −2.5 kb can be down regulated by dexamethasone (13–15). We also know that 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and epidermal growth factor and an oncogenic, mutated form of *ras* often found in primary hepatocarcinomas, can reduce the activities of regulatory elements present in the 5′ end extragenic region of the human AFP gene (16, 17). Transgenic studies indicate that the activities of the mouse AFP regulatory elements can be stimulated during liver regeneration (18), when expression of AFP (19) and of several proto-oncogenes is greatly enhanced (see Ref. 20 for review). The mechanisms underlying these modulations of the AFP regulatory elements are still largely unknown. However, it is quite possible that proto-oncogenes of the Jun family play a key role in some steps of these cascade mechanisms.

The proteins of the Jun family are transcription factors involved in several basic cellular activities which govern differentiation and proliferation and in several signal transduction pathways (see Refs. 21–24 for reviews). These proteins all belong to the class of the basic leucine zipper proteins that bind DNA as dimers. These dimers may be homodimers, but are generally heterodimers formed between two members of the Jun family or mainly in association with members of the Fos family that have a stronger affinity for DNA.

Formation of Jun-Fos (AP1) complexes is the last step in a cascade of events which allows transmission of several types of signals from the membrane to the DNA transcriptional regulatory elements of target genes. For instance, AP1 complex mediates specific transcriptional effects following the activation of membrane receptors (Ras) and that of protein kinases

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¶ Recipient of a postdoctoral fellowship from the French Ministère de la Recherche et de la Technologie. Present address: Dept. of Radiology, The Ohio State University, Columbus, OH 43210.

‖ Recipient of a postdoctoral fellowship from the French Ministère de la Recherche et de la Technologie.

** To whom correspondence and reprint requests should be addressed. Tel.: 33-1-45-07-52-88; Fax: 33-1-45-07-58-90.

[1] The abbreviations used are: AFP, α-fetoprotein; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; C/EBP, CCAAT/enhancer-binding protein; HNF1, hepatocyte nuclear factor 1; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; TRE, TPA-responsive element; LUC, luciferase; GR, glucocorticoid receptor; RXR, retinoic acid X receptor; NF1, nuclear factor 1; kb, kilobase(s); bp, base pair(s).

(protein kinase C) in response to growth factors or chemicals such as phorbol esters (TPA). Several activation steps which involve phosphatases and kinases lead to functional Jun-Fos complexes that act upon binding to DNA sequences whose consensus is TGAC/GTCA (see Refs. 21–24 for reviews).

Jun proteins can also participate in the cross-talk between independent regulatory pathways. For instance, Jun-Fos complexes have been shown to interfere with the cAMP signal transduction pathway because the binding sites for members of the CREB family (consensus TGACGTCA) are very similar to those for the AP1 complex. Jun may also be involved in the negative regulation induced by glucocorticoids. More generally, regulatory interactions of Jun with several steroid-thyroid hormone receptors can occur through mechanisms which are still debated (see Refs. 25–27 for reviews). Jun may also interact directly with some tissue-specific transcription factors of the MyoD (28, 29) and C/EBP families (30).

Very recently, c-Jun was found to participate in the modulation of certain liver-specific regulatory elements, such as the phosphoenolpyruvate carboxykinase promoter (31), the albumin far-upstream enhancer (32), and the hepatocyte nuclear factor 1 (HNF1) promoter (33). It may also participate in the general mechanisms which allow correct development of the liver (34) or modulation of liver gene expression during liver regeneration (35).

The present study was undertaken to determine whether Jun participates directly or indirectly in the modulation of some of the rat AFP regulatory elements in the context of an hepatoma cell line which expresses the AFP gene. Transient transfection experiments were performed to monitor the effects of TPA and overexpressed Jun on the activity of vectors bearing the chloramphenicol acetyltransferase (CAT) gene under the control of the rat AFP promoter region and of its 7 kb of 5′ end-flanking sequences.

The results show that TPA has a strong negative effect on the AFP promoter in the context of the HepG2 hepatoma cells. c-Jun plays a major role in this highly promoter-specific inhibition. Interestingly, c-Jun appears to act indirectly via a mechanism which does not require its binding to a precise DNA region of the AFP promoter and which involves the N-terminal part of the c-Jun protein.

## MATERIALS AND METHODS

### *Plasmids*

*CAT Plasmids*—Plasmids pBL-CAT2 and -3 (36) were used as starting material to construct plasmids bearing the CAT gene under the control of rat AFP or albumin gene promoters. The *SacI*/*Xba*I 1-kb fragment (named UMS) from the upstream region of the mouse c-*mos* gene was cloned just in front of the polylinker region of these plasmids (plasmids pBL-UMS-CAT2 and -3) to prevent the contribution of any spurious initiation of transcription in the plasmid (37). Conventional cloning procedures were used to insert the −197 to +6 (*Bbv*I-*Hpa*II) fragment or the −324 to −15 (*Hin*dIII-*Hae*II) fragment of the rat AFP promoter (38) into the *Hin*dIII-*Bgl*II sites of pBL-UMS-CAT3, yielding plasmids pAFP26-CAT and pAFP-CAT, respectively. The fragment −7200 to −324 (*Eco*RI-*Hin*dIII) of the 5′ end extragenic region of the rat AFP gene was cloned in the polylinker in front of the AFP promoter in plasmid pAFP-CAT to give plasmid pPO123-AFP-CAT. Plasmid pBL-AFP36-CAT was made by cloning the blunted −324 to +6 (*Hin*dIII-*Hpa*II) fragment of the rat AFP promoter into the blunted *Bgl*II site of plasmid pBL-CAT6.

Plasmid pALB-CAT was obtained by cloning the −175 to +15 (*Alu*I-*Hin*cII) region of the rat albumin promoter (39) at the *Hin*dIII-*Bgl*II sites in plasmid pBL-UMS-CAT3.

CAT plasmids bearing the thymidine kinase (TK) promoter of the herpes simplex virus and one copy of a functional or a mutated TPA-responsive element (TRE) of the collagenase gene, pTRE-TK-CAT and pΔTRE-TK-CAT, respectively, have been described previously (40).

*pAFP-LUC Series*—The parent plasmid pAFP-LUC, containing the luciferase (LUC) reporter gene under the control of the AFP promoter, was constructed by inserting the blunted *Hin*dIII-*Hpa*II (−324/+8) fragment of the AFP promoter into the blunted *Bgl*II site of pGL2-basic (Promega). The plasmids pAFP-LUC Δ234, Δ183, Δ155, Δ134, Δ115, Δ67, and Δ49 were generated by progressively deleting 5′ portions of the AFP promoter in the plasmid pAFP-LUC with exonuclease III/mung bean nucleases.

*pTK-LUC and pSV-LUC Series*—To obtain plasmid pTK-LUC, the *Bam*HI-*Bgl*II fragment containing the TK promoter (−105/+51) was excised from pBL-CAT5 and inserted into the *Bgl*II site of pGL2-basic. The pF1 to F4-TK-LUC series was constructed by inserting different fragments of the AFP promoter upstream of the TK promoter in pTK-LUC. The *Bbv*I-*Mae*III (−196/−135), *Mae*III-*Mae*III (−142/−79), and *Bbv*I-*Hin*cII (−196/−52) fragments of the AFP promoter were isolated, blunted, and introduced into the blunted *Nhe*I site of pTK-LUC, generating the pF1, pF2, and pF4-TK-LUC plasmids, respectively. A blunt-ended oligonucleotide encompassing the −85- to −41-bp region of the AFP promoter was cloned into the blunted *Nhe*I site of pTK-LUC to generate plasmid pF3-TK-LUC.

The plasmids pF1-, pF2-, pF3-, and pF4-SV-LUC were constructed in a similar fashion, except that the plasmid pGL2-promoter (Promega) containing the SV40 promoter in front of the luciferase gene was used instead of plasmid pTK-LUC.

The control plasmids pTRE-TK-LUC and pΔTRE-TK-LUC were generated by transferring a fragment carrying the wild type or mutated TPA-responsive element of the collagenase gene and the TK promoter from the pTRE-TK-CAT or pΔTRE-TK-CAT plasmid, respectively, to pGL2-basic. The TRE-TK or ΔTRE-TK fragment was excised with *Hin*dIII-*Bgl*II, blunted, and inserted into the blunted *Bgl*II site of pGL2-basic.

The resulting plasmids were checked by sequencing using the dideoxy method.

*Jun and Fos Expression Vectors*—Vectors allowing the expression of mouse c-Fos (pRSV c-Fos), mouse c-Jun (pRSV c-Jun), a mouse c-Jun lacking its N-terminal activating domain (pRSV c-Jun Δ168) or its leucine zipper region (pRSVc-Jun CDL), and those for JunB and JunD (pRSV JunB, pRSV JunD) were kindly donated by M. Yaniv (41). Vector allowing the expression of human c-Jun was a gift from B. Binetruy and M. Karin. Vectors allowing the expression of human c-Jun proteins with point mutation in the DNA binding domain (pRSV c-Jun-DB3 and pRSV c-Jun-DB4, mutants 12 and 14 in Ref. 42) and that of a chimeric protein made with the N-terminal part of c-Jun (amino acids 1–253) fused to the DNA binding domain of Gal4 (pDB10) were gifts from D. Bohmann (42).

### *Transfection and Transient Expression Experiments*

Human hepatoma cells HepG2 were obtained from the American Type Culture Collection and grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 media containing 10% fetal calf serum. Transfection experiments were performed in 6-cm plastic tissue culture dishes containing about $10^6$ exponentially growing cells using the calcium phosphate method. 2 μg of CAT plasmid were routinely used.

Co-transfection experiments were performed with increasing amounts (0–5 μg) of the expression vectors while keeping the total amount of transfected DNA at 12 μg by adding pUC18 DNA. The precipitate was left in contact with the cells for 4 h. Cells were then submitted to a 20% glycerol shock for 2 min, and the medium was changed. When desired, TPA dissolved in 10 μl of 1% $Me_2SO$ was then added. 48 h later, cells were lysed by three successive freeze-thaw methods in 100 μl of 0.25 M Tris-HCl buffer, pH 7.8. The homogenate was centrifuged for 10 min at 11,000 × *g*, and the clear supernatant was frozen at −20 °C. Protein concentrations were determined by the Bradford method with bovine immunoglobulin as standard (Bio-Rad).

The CAT reaction was routinely monitored on samples containing 50–100 μg of protein from HepG2. CAT activity was measured either after separation of [$^{14}$C]chloramphenicol from the acetyl [$^{14}$C]chloramphenicol by TLC or from the butyryl [$^{14}$C]chloramphenicol by extraction into tetramethyl-*p*-phenylenediamine/xylene (43). Both methods gave the same results in our hands. Under our conditions, the activity of pAFP-CAT in the HepG2 cells was usually about 8–10% of that of plasmid pSV2-CAT, and that of pALB-CAT was 3%. The activity of the promoterless plasmid pBL-UMS-CAT3 was close to background and was not significantly altered by expression of any of the transcription factors used.

The plasmid pCH110, coding for β-galactosidase (2 μg), was used in some control experiments to monitor the efficiency of transfection. This plasmid was not routinely included in the co-transfection experiments because its activity was greatly affected by TPA and by expression of

some of the transcription factors.

HepG2 cells were transfected with the luciferase plasmids in the same way, except that 5 μg of plasmids bearing the luciferase gene were used, together with 5 μg of the pRSV c-Jun expression vector or with 5 μg of pUC18 plasmid. Cells were lysed by adding 400 μl of the Reporter lysis buffer (Promega), incubation for 15 min at room temperature, and scraping. Luciferase activity was recorded for 30 s in a Berthold Lumat LB 9501 luminometer for samples containing 20 μg of protein, using the protocol and reagents given in the Luciferase Assay System (Promega).

All the transfection experiments were repeated 3–8 times, usually in duplicate, with at least two different preparations of the purified plasmids.

### *DNase 1 Footprinting Experiments*

DNA probes for DNase 1 footprinting assays were end-labeled by filling in cohesive ends using [α-$^{32}$P]dATP and Klenow polymerase. Probes were purified by polyacrylamide gel electrophoresis followed by electroelution. A 340-bp DNA probe encompassing the AFP promoter was isolated from a plasmid pBluescript SK+ (Stratagene) carrying the blunted *Hin*dIII-*Hpa*II fragment of the AFP promoter (−324/+8) inserted into the *Eco*RV site. The AFP promoter was labeled on either the antisense or the sense strand using the *Eco*RI or the *Hin*dIII site situated in the pBluescript polylinker. A 227-bp DNA fragment containing the TPA-responsive element of the collagenase promoter and part of the TK promoter was labeled on the sense strand at the *Eco*RI site internal to the TK promoter and subsequently isolated from the pTRE-TK-CAT plasmid by *Nar*I digestion.

Purified recombinant human c-Jun protein was purchased from Promega. DNase 1 footprinting assays were performed as described (8), except that the binding reaction was done in the buffer suggested by Promega, with 1 μg of poly(dI-dC)-poly(dI-dC) as a nonspecific competitor DNA. The specificity of the protections was verified by competition experiments using oligonucleotides carrying an AP1 consensus binding site or unrelated sequences.

## RESULTS

*Inhibition of the AFP Promoter in HepG2 Cells by TPA*—The action of TPA on the activities of AFP regulatory elements in the promoter of the rat AFP gene and in its 7-kb 5′ end extragenic region was examined by monitoring the CAT activity of plasmids pPO123-AFP-CAT and pAFP-CAT transiently expressed in HepG2 cells. The activities of both plasmids were greatly reduced (75–88%) when HepG2 cells were treated with TPA (Fig. 1*A*). The basal activity of the pPO123-AFP-CAT plasmid was much more higher (about 8-fold) than that of pAFP-CAT, in agreement with the fact it contains several enhancer elements acting on the AFP promoter (14). The effect of TPA on the activity of several other CAT plasmids was also assessed under the same conditions, as controls. The promoterless plasmid pBL-UMS-CAT had very little CAT activity (near background) that was not altered by TPA. The activity of the plasmid pTRE-TK-CAT, which contains one copy of the TPA-responsive element (TRE) of the collagenase gene in front of the TK promoter, was stimulated by TPA, as expected. In contrast, the activity of pΔTRE-TK-CAT, which contains the TRE element with a 2-bp deletion that greatly reduces the binding of Jun, was not affected (Fig. 1*A*).

Although we could not exclude that the region from −7000 to −330 of the rat AFP gene does not contain negative TPA responsive elements, we focused on the AFP promoter. Fig. 1*B* shows that the negative effect of TPA on the AFP promoter activity was dose-dependent. This effect also appeared to be specific to the AFP promoter, since the activity of pALB-CAT, which contains the albumin promoter region (−175 to +15) instead of the AFP promoter in the same pBL-UMS-CAT vector, was unaffected by TPA over the same range of doses.

Thus, TPA specifically decreases the activity of the AFP promoter, in HepG2 cells, but not that of the albumin promoter.

*Negative Regulation of the AFP Promoter in HepG2 Cells by Jun Transcription Factors*—The effect of Jun on the AFP promoter activity was checked by co-transfecting HepG2 cells with

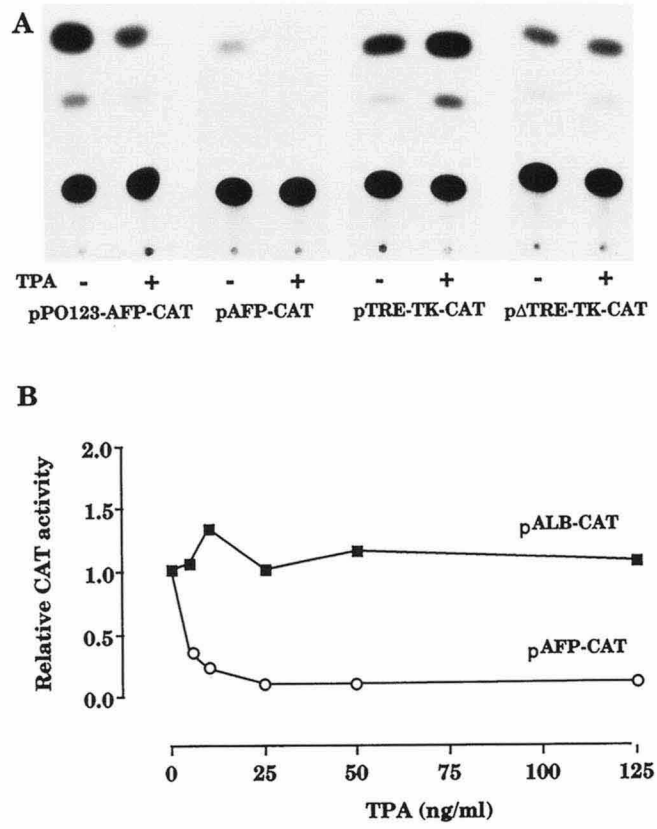


FIG. 1. **Negative modulation of the rat AFP gene promoter by TPA in HepG2 hepatoma cells.** *A*, HepG2 cells were transfected with 2 μg of CAT plasmid bearing the rat AFP promoter and 7 kb of the rat AFP gene 5′ end extragenic region (*pPO123-AFP-CAT*) or the rat AFP promoter alone (*p-AFP-CAT*), 2 μg of plasmids containing the TK promoter, and a single copy of the TPA responsive element (TRE) of the collagenase gene (*pTRE-TK-CAT*) or a mutated TRE (*pΔTRE-TK-CAT*) were used as controls within the same batch of experiments. CAT activities were monitored 48 h later for HepG2 cells treated with 50 ng of TPA in 10 μl of 1% $Me_2SO$(+) or 10 μl of 1% $Me_2SO$ alone (−). *B*, 2 μg of CAT plasmids bearing the AFP promoter (*pAFP-CAT*) or the albumin promoter (*pALB-CAT*) were transfected into HepG2 cells treated with increasing amounts of TPA in 1% $Me_2SO$ or with 1% $Me_2SO$ alone. CAT values corresponding to plasmid pAFP-CAT (○) or pALB-CAT (■) are expressed with reference to those obtained with the corresponding plasmids in the absence of TPA.

the pAFP-CAT plasmid plus increasing amounts of plasmid pRSV c-Jun, which allows expression of the mouse c-*jun* protooncogene. The activity of plasmid pAFP-CAT was lowered in a manner dependent on the amount of the c-Jun expression vector (Fig. 2). It was inhibited to 12–15% of its basal value. Similar results were obtained with a pRSV vector allowing expression of the human c-Jun protein (data not shown).

The strong negative effect of c-Jun on the AFP promoter was highly specific. The expression of c-Jun did not significantly alter the CAT activity of the plasmid bearing the albumin promoter and had, as expected, a positive effect on the pTRE-TK-CAT plasmid carrying the TRE of the collagenase gene. The specificity of the response of these plasmids also indicated that the negative effect of c-Jun on the AFP promoter-containing vectors did not result from general blocking of RNA-PolII-dependent transcription machinery or from unrelated AP1 sites present in the plasmid (46).

The promoter specificity and magnitude of the response observed with c-Jun were the same as that induced by TPA. This strongly suggested that the effect of TPA is mainly mediated by c-Jun under our conditions. These results clearly demonstrate that c-Jun can participate in the modulation of the AFP promoter activity in HepG2 hepatoma cells.

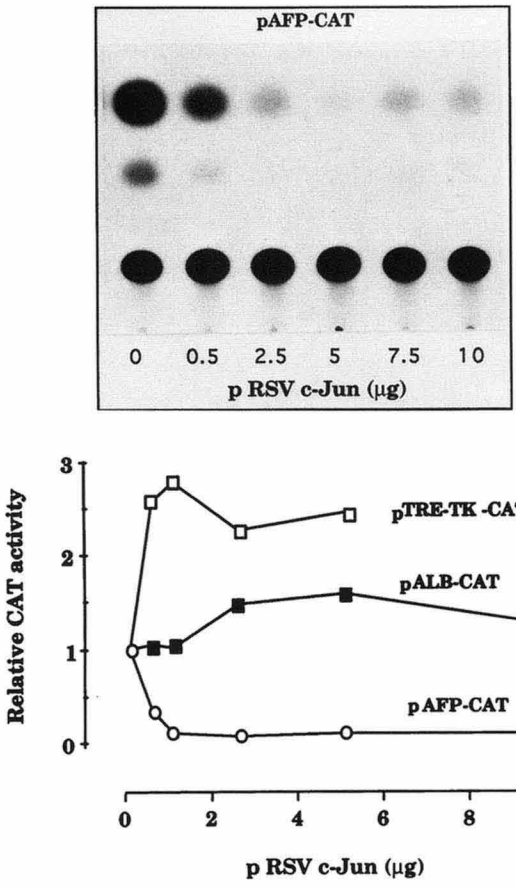


FIG. 2. **c-Jun down-regulates the rat AFP promoter activity in HepG2 hepatoma cells.** HepG2 cells were transfected with 2 μg of CAT plasmid bearing the rat AFP promoter (pAFP-CAT), the rat albumin promoter (pALB-CAT), or the thymidine kinase promoter of the herpes simplex virus (TK) and the TRE from the collagenase gene (pTRE-TK-CAT), together with increasing amounts of a mouse c-Jun expression vector (pRSV c-Jun). The total amount of transfected DNA was kept constant at 12 μg by addition of pUC18. CAT activities were measured 48 h after transfection. The *upper part* of the figure shows the variation of the activity of the pAFP-CAT plasmid as a function of increasing amounts of the pRSV c-Jun plasmid. In the *lower part* of the figure, activities of the pAFP-CAT plasmid (○), of the pALB-CAT plasmid (■), and of the pTRE-TK-CAT plasmid (□) are expressed with reference to those of the corresponding plasmids in the absence of c-Jun expression vector.

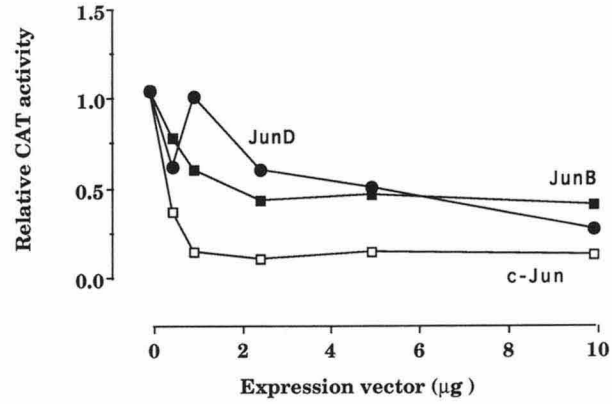


FIG. 3. **Effects of mouse JunB and mouse JunD on the rat AFP promoter activity in HepG2 hepatoma cells.** HepG2 cells were transfected with 2 μg of the CAT plasmid bearing the rat AFP promoter region (pAFP-CAT) together with increasing amounts of plasmids allowing expression of mouse c-Jun (□), JunB (■), or JunD (●). The total amount of transfected DNA was kept constant at 12 μg by addition of pUC18. CAT activities were measured 48 h later and are expressed with reference to the activity of the pAFP-CAT plasmid in the absence of Jun expression vectors.

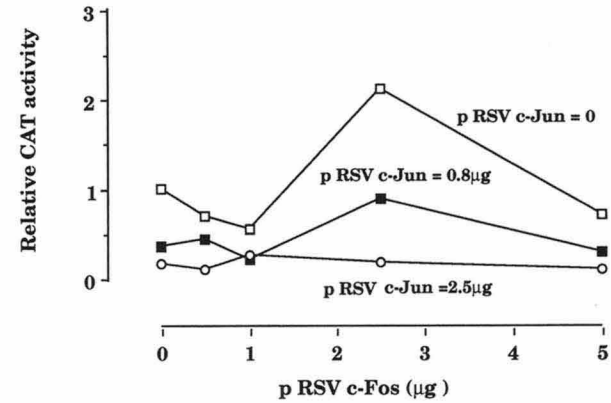


FIG. 4. **Effects of c-Fos or combined c-Fos-c-Jun expression on the rat AFP promoter activity in HepG2 hepatoma cells.** HepG2 cells were transfected with 2 μg of the CAT plasmid bearing the rat AFP promoter (pAFP-CAT) together with increasing amounts of a mouse c-Fos expression vector (pRSV c-Fos) alone (□) or in the presence of 0,8 μg (■) or 2,5 μg (○) of a mouse c-Jun expression vector (pRSV c-Jun). The total amount of transfected DNA was kept constant at 12 μg by addition of pUC18. CAT values were measured 48 h later and are expressed with reference to that of the pAFP CAT plasmid in the absence of any expression vector.

We observed that TPA treatment of the cells can further enhance the down-regulating effect of the overexpressed Jun (data not shown). This strongly suggests that the TPA-mediated stimulation of the cellular protein kinase C induces modifications (phosphorylation) of the overexpressed c-Jun which result in the reinforcemenet of its negative action on the AFP promoter. Thus, although the overexpressed c-Jun may not be present in its fully active form in the absence of protein kinase C stimulation, it acts in the same way as a cellular Jun molecule that has been "activated" by TPA.

We used a similar approach to show that two other members of the Jun family of transcription factors, JunB and JunD, also acted on the AFP promoter and decreased its activity (Fig. 3). However, assuming that the same quantities of functional proteins were produced by the three expression vectors, JunB and JunD were poorer inhibitors of the AFP promoter in HepG2 cells than was c-Jun.

Since Jun usually binds to DNA as a heterodimer with Fos, we tested the effect of the c-*fos* proto-oncogene alone and in combination with different amounts of c-Jun on the AFP promoter activity. HepG2 cells were co-transfected with the pAFP-CAT plasmid and increasing amounts of c-Fos expression vector. The influence of different c-Fos:c-Jun ratios was examined by using the c-Fos expression vector alone or with two quantities of the c-Jun expression vector (Fig. 4). c-Fos expression alone has very little, if any, negative effect on the AFP promoter activity. More importantly, c-Fos never potentiated the negative effect of c-Jun on the AFP promoter. Fos even seemed to counteract c-Jun at some Fos:Jun ratios. This suggests that c-Jun, and not a c-Jun-c-Fos complex, is mainly responsible for down-regulating the AFP promoter in HepG2 cells.

*The Repression of AFP Promoter Activity Does Not Require Binding of c-Jun to DNA and Involves the N-terminal Part of c-Jun*—We determined whether c-Jun can bind to the AFP promoter by DNase 1 footprinting experiments with purified recombinant human c-Jun (Fig. 5). A probe containing the TRE-TK promoter was used as a control. Purified c-Jun did not specifically bind to either of the two strands of the rat AFP promoter under the conditions where a clear footprint was observed in the TRE region of the TRE-TK promoter (Fig. 5).

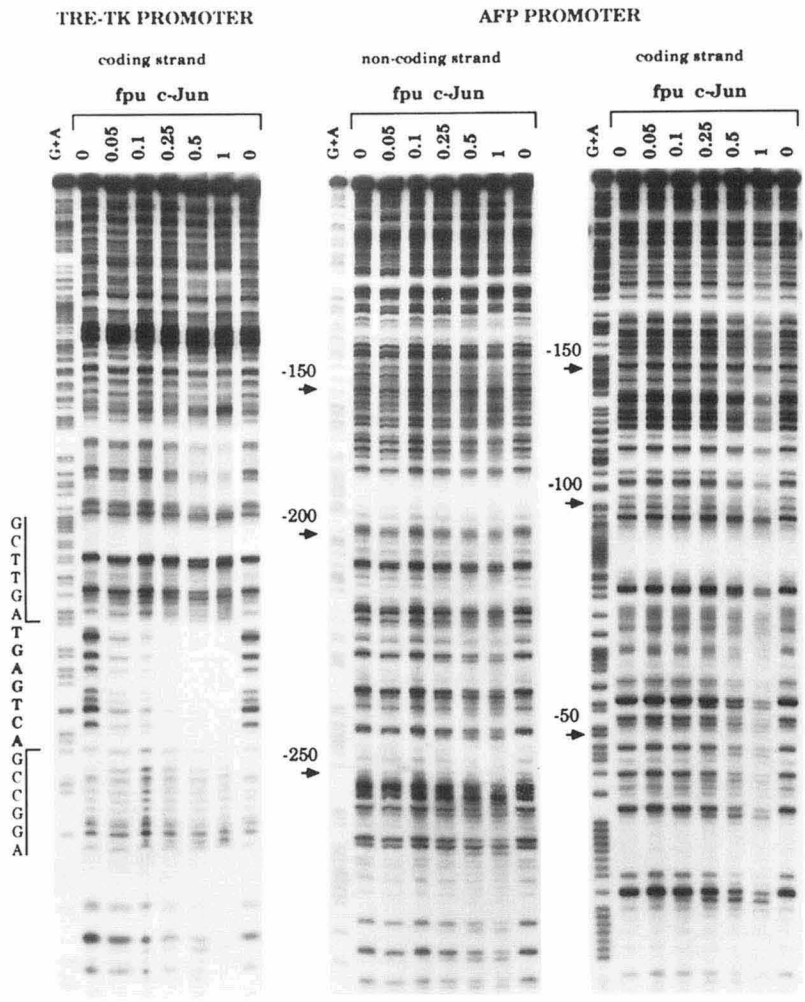


FIG. 5. **Purified human c-Jun does not bind with high affinity to the rat AFP promoter.** 1 ng of double-stranded DNA fragments corresponding to the $^{32}$P-labeled noncoding strand (*middle panel*) or coding strand (*right panel*) of the rat AFP promoter and to the coding strand (*left panel*) of a construct containing the TRE of the collagenase gene cloned in front of the TK promoter (from pTRE-TK-CAT) were incubated without (0) or with increasing amounts of purified recombinant human c-Jun protein (up to 1 footprinting unit) and then subjected to DNase 1 digestion as described under "Materials and Methods." Aliquots of G + A Maxam and Gilbert reactions performed on each of the labeled fragments were run as molecular weight markers on the denaturating acrylamide gel. Numbering of the AFP fragment is from the transcription start site (38). The nucleotide sequence of the protected TRE element in the TRE-TK probe is written in the *left margin*. The specificity of the protections was verified by competition experiments using oligonucleotides carrying an AP1 consensus binding site or unrelated sequences.

This indicated that c-Jun cannot bind to the rat AFP promoter with high affinity.

To determine which part of the c-Jun protein is required for the negative regulation of the AFP promoter, we used vectors allowing the expression of mutated c-Jun proteins. The activity of two mutants of c-Jun ($JunDB_3$ and $JunDB_4$) with point mutations in the DNA binding domain which greatly lowered their affinity for a TRE binding site (42), was monitored. We also tested mutants c-JunΔ168 and c-Jun CDL (41) from which the activating domain or the Leucine Zipper region had been deleted, respectively (Fig. 6). Mutants lacking the activating domain or the leucine zipper domain had lost their capacity to repress the AFP promoter activity. In contrast, the two c-Jun proteins mutated in their DNA binding domain (DB3 and DB4) were still able to down regulate the AFP promoter (Fig. 6). Hence, the integrity of the DNA binding domain of Jun is not required for the c-Jun protein to exert its negative effect on the AFP promoter in HepG2 cells.

The absence of a high affinity binding site for c-Jun on the AFP promoter, plus the fact that the integrity of the c-Jun DNA binding domain is not required for the negative effect, strongly suggests that the repressive action of Jun on the AFP promoter does not involve a direct binding of Jun to the AFP promoter. This hypothesis led us to test the effect of a chimeric protein made of the N-terminal part of c-Jun, (amino acids 1–253) which contains the Jun activating domain, fused to the DNA binding domain of the Gal4 transactivator (42). The chimeric Jun-Gal4 protein, expressed from plasmid pDB10, strongly repressed the AFP promoter (Fig. 7). By opposition, plasmid

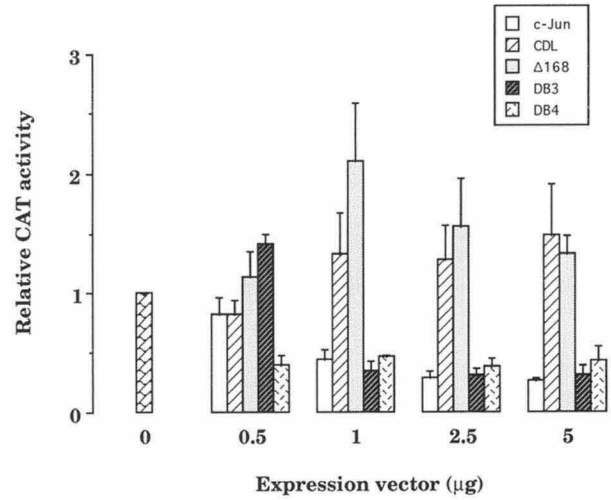


FIG. 6. **Effects of mutated c-Jun on the rat AFP promoter activity in HepG2 hepatoma cells.** HepG2 cells were transfected with 2 μg of the pAFP26-CAT plasmid bearing the AFP promoter, alone (0) or together with increasing amounts of vectors allowing expression of a wild type mouse c-Jun protein (pRSV c-Jun) or of a c-Jun protein deleted from the activating domain (pRSV CDL c-Jun) or from the leucine zipper (pRSV Δ168 c-Jun) or bearing point mutations in the DNA binding domain (pRSV DB3 c-Jun and pRSV DB4 c-Jun). Total amount of transfected DNA was kept constant to 12 μg by addition of pUC18 DNA. CAT values were measured 48 h later and are expressed with reference to the activity of the pAFP26-CAT plasmid in the absence of any expression vector. Results are given as the mean of 4 independent experiments. *Bars* represent the mean ± standard error.

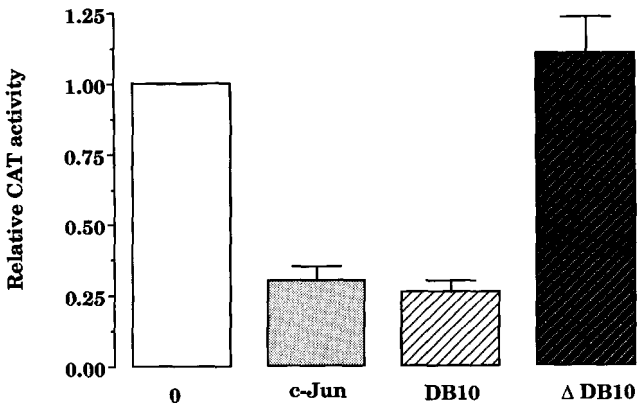


FIG. 7. **The activating domain of c-Jun is sufficient for down-regulating the rat AFP promoter in HepG2 hepatoma cells.** HepG2 cells were transfected with 5 μg of the pAFP26-CAT plasmid bearing the AFP promoter, alone (0) or together with 1 μg of a vector allowing expression of a wild type mouse c-Jun protein (pRSV c-Jun) or of a chimeric protein made of the N-terminal activating domain of c-Jun linked to the DNA binding domain of the Gal4 protein (pDB10). pΔDB10 is the same plasmid as pDB10 except that the DNA sequences corresponding to the N-terminal part of c-Jun have been deleted. Total amount of transfected DNA was kept constant at 10 μg by addition of pUC18 DNA. CAT values were measured 48 h later and are expressed with reference to the activity of the pAFP26-CAT plasmid in the absence of any expression vector. Results are given as the mean of 4 independent experiments. *Bars* represent the mean ± standard error.

pΔDB10, which was obtained from pDB10 by deleting only the DNA sequences coding for the N-terminal part of Jun, had no effect on the AFP promoter (Fig. 7). These results clearly indicated that the N-terminal part of Jun, which contains the activating domain, is responsible for the down-regulation of the AFP promoter. Similar results were obtained with another chimeric protein containing the N-terminal part of c-Jun (amino acids 1–193) fused to the DNA binding domain of the growth hormone transcription factor 1 (data not shown).

This finding prompted us to use a functional assay to locate the region of the AFP promoter which is the target for the indirect c-Jun action. We constructed a series of vectors containing the luciferase gene under the control of the AFP promoter that had been progressively deleted from its 5′ end by exonuclease III. They were introduced into the HepG2 cells alone or together with the c-Jun expression vector. Deleting the AFP promoter from −336 to −134 resulted in a gradual decrease in its activity (Fig. 8*A*). The background level was reached with the deletion at −115. This decrease in the activity reflects the progressive loss of binding sites, at least for C/EBP at −280 (8), for the fetoprotein transcription factor (FTF) (11) and the glucocorticoid receptor (GR) in the −160 region (13), and those for the retinoic acid X receptor (RXR) and COUP at −135 (44). This deletion analysis also confirmed that the −120 region, to which HNF1, nuclear factor 1 (NF1), and the C/EBPs can bind, is crucial for the AFP promoter functioning (5, 7, 8, 12).

The luciferase activities of the plasmids carrying the intact −324 AFP promoter and its −234, −183, −155, and −134 deletion derivatives were all greatly lowered by expression of the c-Jun protein. Consequently, any region between −324 and −134 is clearly not needed for the negative effect. However, they can always be targets for a c-Jun action. The results also strongly suggested that one or more elements in the region between −134 and +6 are involved in the repression of the AFP promoter activity by c-Jun.

We used another approach to locate the region(s) where c-Jun exerted its action. Overlapping fragments of the AFP promoter known to bind regulatory proteins were cloned into luciferase plasmids containing the TK promoter instead of the AFP promoter. Each of these fragments, −196/−52, −196/−135, −142/−79, −85/−41, slightly stimulated the TK promoter activity in the HepG2 cells (Fig. 8*B*). However, the activity of none of these F1-to-F4 TK plasmids was blocked by overexpression of c-Jun (Fig. 8*B*). This indicated that none of these fragments, when present in a single copy, is able to confer the c-Jun negative regulation on the TK promoter.

The F1 to F4 fragments of the rat AFP promoter were also cloned into luciferase plasmids containing the SV40 promoter instead of the TK promoter and used in transient expression experiments in HepG2 cells. None of these F1 to F4 fragments inhibited the stimulatory effect that c-Jun exerted on the basic pSV-LUC plasmid upon binding to the AP1 site present in the SV40 promoter (data not shown).

## DISCUSSION

The present study demonstrates that TPA and members of the Jun family of transcription factors can specifically block the activity of the rat AFP promoter in HepG2 hepatoma cells. These effects are specific to the AFP promoter, as neither the liver-specific albumin promoter nor the ubiquitously active TK promoter were modulated by TPA or c-Jun when assayed under the same conditions. This specificity excludes the trivial risks of "squelching" or the use of a cryptic Jun responsive element in the vector (45, 46).

c-Jun was found to be more potent than JunB or JunD in down-regulating the AFP promoter. Other examples of such a hierarchy in the members of the c-Jun family have been reported for several biological actions (47–49). c-Fos never acted in synergy with c-Jun to block the AFP promoter activity.

The specificity of the negative response of the rat AFP promoter to members of the Jun family is reminiscent of an elegant study which showed that Ras can down-regulate the human AFP promoter in HuH7 hepatoma cells, but not the albumin promoter (17). As in our case, the exact region of the human AFP promoter involved in this response to Ras could not be precisely mapped. The results suggested that the suppressive effect of Ras is mediated by one or more elements within the 169-bp region upstream of the transcription initiation site. We now know that one of the effects of Ras is to stimulate the c-Jun activity in several cell types. It may well be that the effects of Ras on the AFP promoter involve c-Jun. Supporting this hypothesis, the same group has very recently reported that c-Jun can down-regulate the AFP promoter in HuH7 cells (50). These results therefore support the notion that a G protein-linked signaling cascade, or other pathways acting through the Jun family of transcription factors, might be involved in the differential regulation of the AFP and albumin promoters during normal development and in liver cancer. We have, similarly, shown that the AFP promoter can use several strategies, including the use of HNF1β rather than HNF1α and competition between HNF1 and NF1, to specifically regulate its activity in a way different from that of the albumin promoter (12).

The mechanisms leading to negative regulation in eukaryotes may be of several types and are very often complex (see Ref. 51 for review). Those involving c-Jun are well documented (see Refs. 25–27 for reviews). Here, it appears that the down-regulation of the AFP promoter by c-Jun does not involve the binding of c-Jun to a precise region of the AFP promoter. Our DNase 1 footprinting experiments showed no high affinity binding site for c-Jun on the AFP promoter. The transfection experiments showed that c-Jun proteins, mutated in their DNA binding domains, retained the ability to down-regulate the AFP promoter. That c-Fos did not enhance the c-Jun effect is in agreement with these findings. Indeed, if binding of Jun to

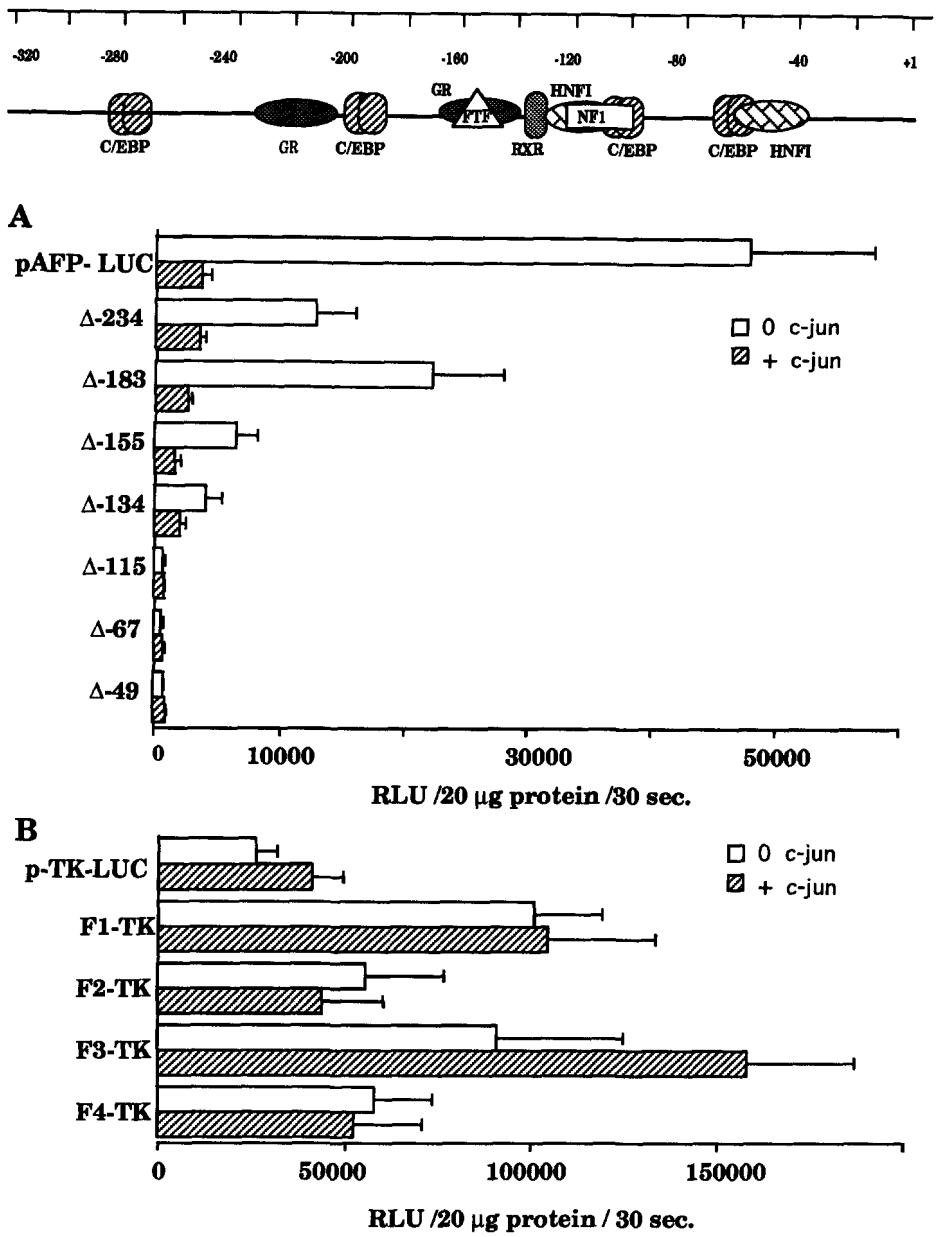


FIG. 8. **Effects of c-Jun on the activity of deletion mutants and of subfragments of the rat AFP promoter in HepG2 hepatoma cells.** The *upper part* of the figure shows a scheme of the rat AFP promoter with the position of binding sites for transcription factors which have been previously mapped using gel-shift or footprinting experiments. *A*, HepG2 cells were transfected with 5 μg of a plasmid bearing the luciferase gene under the control of the rat AFP promoter region in its whole −330/+8 (pAFP-LUC) or truncated at −234, −183, −155, −134, −115, −67, −49, in the absence (□) or in the presence (▨) of 5 μg of the pRSV c-Jun vector allowing expression of the mouse c-Jun. The total amount of transfected DNA was kept constant at 10 μg by addition of pUC18. Luciferase activity was determined 48 h later as described under "Materials and Methods," and values are expressed as relative light units (*RLU*) integrated for 30 s for samples containing 20 μg of protein. Results are given as the mean of 6 to 9 independent experiments. *Bars* represent the mean ± standard error. *B*, one copy of fragment −196/−135 (F1), −142/−79 (F2), −85/−41 (F3), or −196/−52 (F4) of the rat AFP promoter was cloned in front of the TK promoter in the pGL2-TK-LUC plasmid. The effect of c-Jun was checked on 5 μg of each of the resulting F-TK plasmids introduced into HepG2 cells in the absence (□) or presence (▨) of the pRSV c-Jun vector exactly as described above.

DNA were involved, one might have expected a synergy between c-Fos and c-Jun in repressing the AFP promoter because the affinity of the Jun-Fos heterodimer for DNA is stronger than that of the Jun homodimer. Lastly, the experiments with the Jun-Gal4 chimera clearly showed that the N-terminal part of the c-Jun protein which contains the activating domain is sufficient for the negative effect.

We cannot definitively rule out the possibility that TPA or c-Jun stimulate the expression/activity of an inhibitory factor or repress that of a *trans*-activator. However, we believe that the effect of Jun on the AFP promoter is mainly mediated by negative regulatory interactions between Jun and another transcription factor. Such interactions may involve interference with binding to DNA or interference with transcriptional activation. Since Jun does not need to be bound to DNA for repression of the AFP promoter activity, it is unlikely that the mechanism entails interference with binding to DNA. This down-regulation could require protein-protein interactions between Jun and another(other) transcription factor(s) which favor the functioning of the AFP promoter. The fact that c-Fos could not potentiate the effect of c-Jun, but even seemed to antagonize c-Jun action at some c-Jun:c-Fos ratios, favors this hypothesis. Fos overexpression would result in the trapping of c-Jun, which would thus be no longer available to interact with a positive *trans*-acting factor on the AFP promoter and counteract its stimulatory effect.

Other examples of negative regulation by Jun which do not require the binding of Jun to DNA, but are mediated through interaction of the N-terminal activating domain of Jun with MyoD or cardiac co-activators have been reported (28, 29).

Jun is known to also counteract the action of several steroid/thyroid hormone receptors: the glucocorticoid (GR), progesterone, and estrogen receptors, the thyroid hormone receptor, and the retinoid receptors RAR/RXR (see Refs. 25–27 for reviews). GR and RXR, therefore, might have been among the best candidates for negative interaction with c-Jun on the AFP promoter. It has been shown that the GR and that the RXR can bind to regions at −160 and −135 of the rat AFP promoter, respectively. Upon addition of their respective ligands, GR down-regulates (13, 14, 52, 53) and RXR stimulates (44) the activity of the AFP promoter. Our experimental results, however, do not confer the major role to interactions between Jun

and the GR or the RXR in the down-regulation of the AFP promoter by Jun. Deletions of the regions bearing their binding sites on the AFP promoter let the remaining promoter still able to negatively respond to Jun. This indicates that Jun can act downstream in the AFP promoter upon interaction with protein(s) other than the GR or the RXR. It is, however, possible that interactions between Jun and the GR or RXR might play a significant role in the molecular mechanisms which govern the response of the AFP promoter to glucocorticoids or retinoids.

The *trans*-activator target for the Jun repression of the AFP promoter was not identified because our different approaches using the luciferase gene under control of regions of the rat AFP promoter failed to point out a single short DNA region of the AFP promoter where Jun exerts its regulatory effect. The negative effect of c-Jun we observed on the 330-bp fragment of the AFP promoter may also reflect the sum of several negative effects which take place at different positions on the AFP promoter. There may be a negative interaction of Jun with one of the components of the general transcriptional machinery as just shown for Fos (54). A strong specificity, conferred by the context of the proximal AFP promoter region should be required however. A parallel can be drawn with studies showing that another DNA-binding protein, p53 protein implicated in the control of cell proliferation and tumor progression, can specifically down-regulate various promoters (55). The identification of *cis* targets of this negative regulation by p53 has also proved to be difficult. In fact, minimal promoters containing little more than a TATA box can be down-regulated. Direct interaction between p53 and factors of the general machinery of transcription may be responsible for this negative effect (see Ref. 51 for review). Here again, the exact mechanism is not known and the question of how the promoter specificity of the response is achieved remains open.

Answers to these questions are of crucial importance for a better understanding of how powerful and versatile proteins such as the Jun proteins are involved in cross-talk between tightly regulated pathways so as to achieve specific goals within a given cell and promoter context.

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REFERENCES

1. Sell, S., and Becker, F. F. (1978) *J. Natl. Cancer Inst.* **60,** 19–26
2. Tamaoki, T., and Fausto, N. (1984) *Recombinant DNA Cell Proliferation*, pp. 145–168, Academic Press, Inc., Orlando, FL
3. Nahon, J. L. (1987) *Biochimie (Paris)* **69,** 445–459
4. Papaconstantinou, J., Rabek, J. P., and Zhang, D.-E. (1990) *Dev. Growth & Differ.* **32,** 205–216
5. Jose-Estanyol, M., and Danan, J.-L. (1988) *J. Biol. Chem.* **263,** 10865–10871
6. Jose-Estanyol, M., Poliard, A., Foiret, D., and Danan J.-L. (1989) *Eur. J. Biochem.* **181,** 761–766
7. Feuerman, M. H., Godbout, R., Ingram, R. S., and Tilghman, S. M. (1989) *Mol. Cell. Biol.* **9,** 4204–4212
8. Thomassin, H., Hamel, D., Bernier, D., Guertin, M., and Bélanger, L. (1992) *Nucleic Acids Res.* **20,** 3091–3098
9. Morinaga, T., Yasuda, H., Hashimoto, T., Higashio, K., and Tamaoki, T. (1991) *Mol. Cell. Biol.* **11,** 6041–6049
10. Zhang, D.-E., Hoyt, P. R., and Papaconstantinou, J. (1990) *J. Biol. Chem.* **265,** 3382–3391
11. Bernier, D., Thomassin, H., Allard, D., Guertin, M., Hamel, D., Blaquière, M., Beauchemin, M., LaRue, H., Estable-Puig, M., and Bélanger, L. (1993) *Mol. Cell. Biol.* **13,** 1619–1633
12. Bois-Joyeux, B., and Danan, J.-L. (1994) *Biochem. J.* **301,** 49–55
13. Guertin, M., LaRue, H., Bernier, D., Wrange, O., Chevrette, M., Gingras, M.-C., and Bélanger, L. (1988) *Mol. Cell. Biol.* **8,** 1398–1407
14. Poliard, A., Bakkali, L., Poiret, M., Foiret, D., and Danan, J.-L. (1990) *J. Biol. Chem.* **265,** 2137–2141
15. Turcotte, B., Meyer, M.-E., Bocquel, M.-T., Bélanger, L., and Chambon, P. (1990) *Mol. Cell. Biol.* **10,** 5002–5006
16. Nakata, K., Motomura, M., Nakabayashi, H., Ido, A., and Tamaoki T. (1992) *J. Biol. Chem.* **267,** 1331–1334
17. Nakao, K., Lawless, D., Ohe, Y., Miyao, Y., Nakabayashi, H., Kamiya, H., Miura, K., Ohtsuka, E., and Tamaoki, T. (1990) *Mol. Cell. Biol.* **10,** 1461–1469
18. Hammer, R. E., Krumlauf, R., Camper, S., A., Brinster, R. L., and Tilghman, S. M. (1987) *Science* **235,** 53–58
19. Bernuau, D., Poliard, A., and Feldmann, G. (1988) *Hepatology (Baltimore)* **8,** 997–1005
20. Fausto, N., and Mead, J. E. (1989) *Lab. Invest.* **60,** 4–13
21. Gutman, A., and Wasylyk B. (1991) *Trends Genet.* **7,** 49–54
22. Karin, M. (1992) *FASEB J.* **6,** 2581–2590
23. Curran, T. (1992) *Tohoku J. Exp. Med.* **168,** 169–174
24. Radler-Pohl, A., Gebel, S., Sachsenmaier, C., König, H., Krämer, M., Oehler, T., Streile, M., Ponta, H., Rapp, U., Rahmsdorf, H. J., Cato, A. C. B., Angel, P., and Herrlich, P. (1993) *Ann. N. Y. Acad. Sci.* **684,** 127–148
25. Gronemeyer, H. (1992) *FASEB J.* **6,** 2524–2529
26. Pfahl, M. (1993) *Endocrine Rev.* **14,** 651–658
27. Tsai, M.-J., and O'Malley, B. (1994) *Annu. Rev. Biochem.* **63,** 451–486
28. Li, L., Chambard, J.-C., Karin, M., and Olson, E. N. (1992) *Genes & Dev.* **6,** 676–689
29. McBride, K., Robitaille, L., Tremblay, S., Argentin, S., and Nemer, M. (1993) *Mol. Cell. Biol.* **13,** 600–612
30. Hsu, W., Kerppola, T., Chen, P.-L., Curran, T., and Chen-Kiang, S. (1994) *Mol. Cell. Biol.* **14,** 268–276
31. Gurney, A. L., Park, E. A., Giralt, M., Liu, J., and Hanson, R. W. (1992) *J. Biol. Chem.* **267,** 18133–18139
32. Hu, J., and Isom, H. (1994) *Mol. Cell. Biol.* **14,** 1531–1543
33. Kuo, C. J., Conley, P. B., Chen, L., Sladek, F. M., Darnell, J. E., and Crabtree, G. (1992) *Nature* **355,** 457–461
34. Hilberg, H., Aguzzi, A., Howells, N., and Wagner E. F. (1993) *Nature* **365,** 179–181
35. Hsu, J.-C., Bravo, R., and Taub, R. (1992) *Mol. Cell. Biol.* **12,** 4654–4665
36. Luckow, B. L., and Schutz, G. (1987) *Nucleic Acids Res.* **15,** 5490
37. Wood, T. G., McGeady, M. L., Baroudy, B. M., Blain, D. G., and Van de Warde, G. F. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 7817–7821
38. Nahon, J. L., Danan, J. L., Poiret, M., Tratner, I., José-Estanyol, M., and Sala-Trépat, J. M. (1987) *J. Biol. Chem.* **262,** 12479–12487
39. Sargent, T. D., Jagodzinski, L. L., Yang, M., and Bonner, J. (1981) *Mol. Cell. Biol.* **1,** 871–883
40. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P., and Karin, M. (1987) *Cell* **49,** 729–739
41. Hirai, S.-I., Bourachot, B., and Yaniv, M. (1990) *Oncogene* **5,** 39–46
42. Bohmann, D., and Tjian, R. (1989) *Cell* **59,** 709–717
43. Seed, B., and Sheen, J.-Y. (1988) *Gene (Amst.)* **67,** 271–277
44. Liu, Y., and Chiu, J.-F. (1994) *Nucleic Acids Res.* **22,** 1079–1086
45. Shemshedini, L., Knauthe, R., Sassone-Corsi, P., Pornon, A., and Gronemeyer, H. (1991) *EMBO J.* **10,** 3839–3849
46. Kushner, P. J., Baxter, J. D., Duncan, K. G., Lopez, G. N., Schaufele, F., Uht, R. M., Webb, P., and West, L. W. (1994) *Mol. Endocrinol.* **8,** 405–407
47. Rysek, R.-P., and Bravo, R. (1991) *Oncogene* **6,** 533–542
48. Doucas, V., Spyrou, G., and Yaniv, M. (1991) *EMBO J.* **10,** 2237–2245
49. Castellazzi, M., Spyrou, Y., La Vista, N., Dangy, J. P., Piu, F., Yaniv, M., and Brun, G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 8890–8894
50. Saegusa, M., Ido, A., Nakabayashi, H., Sakai, M., and Tamaoki, T. (1994) *J. Tumor Marker Oncol.* **9,** 29–33
51. Clark, A. R., and Docherty, K. (1993) *Biochem. J.* **296,** 521–541
52. Guertin, M., Baril, P., Bartkowiak, J., Anderson, A., and Bélanger, L. (1983) *Biochemistry* **22,** 4296–4302
53. Zhang, X.-K., Dong, J.-M., and Chiu, J.-F. (1991) *J. Biol. Chem.* **266,** 8248–8254
54. Metz, R., Bannister, A. J., Sutherland, J. A., Hagemeier, C., O'Rourke, E., Cook, A., Bravo, R., and Kouzarides, T. (1994) *Mol. Cell. Biol.* **14,** 6021–6029
55. Ginsberg, D., Mechta, F., Yaniv, M., and Oren, O. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 9979–9983