C/EBP and c-JUN proteins activate the proximal enhancer of the developmentally regulated α-fetoprotein gene

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ABSTRACT The expression of the α-fetoprotein (AFP) gene is developmentally regulated. Active transcription of this gene depends on a proximal enhancer sequence located between positions D-203 bp and -81 bp, upstream the initiation site. This enhancer contains several putative binding sites for transcription factors. By transfection experiments, we showed that the enhancer activity can be driven by interactions with two regulatory factors, namely C/EBP and c-JUN.

KEY WORDS: *α-fetoprotein, C/EBP, c-JUN*

Introduction

Developmental decisions are likely to be taken by quantitative and qualitative modifications of transcriptional factor activities, and the maintenance of cell type specificity is certainly dependent on the expression of particular subsets of transcription factor genes. The understanding of developmental and tissue-specific regulation of gene expression thus requires the study of the interactions between these regulatory proteins and their target genes.

An attractive model for analyzing mechanisms of transcriptional control during development and differentiation is the α-fetoprotein (AFP) gene. Indeed, expression of this gene is highly tissue-specific and is developmentally controlled: the gene is active in the visceral endoderm, the yolk sac, the fetal gut and the fetal liver. After birth, the AFP gene is shut off (for reviews, see Abelev, 1971; Sell and Becker, 1978; Ruoslahti and Sepällä, 1979; Belanger *et al.*, 1983). In the adult, AFP is only detected in regenerating liver and tumors (hepatomas and teratocarcinomas). This pattern of expression is regulated at the transcriptional level (Tilghman and Belayew, 1982; Nahon *et al.*, 1987; Guertin *et al.*, 1989) and involves several cis-acting control regions. In this respect, three distal enhancers and a cell type-specific promoter region have been identified in the mouse AFP gene (Godbout *et al.*, 1986 and 1988; Widen and Papaconstantinou, 1986, 1987; Camper and Tilghman, 1989; Feuerman *et al.*, 1989). Similar regulatory regions were found in the human and rat genes (Watanabe *et al.*, 1987; Guertin *et al.*, 1989; Poliard *et al.*, 1990). In addition, specific expression of the AFP gene depends on a proximal enhancer sequence located between positions -203 bp and -81 bp, upstream the gene (Molné *et al.*, 1989; Houart *et al.*, 1990). This proximal enhancer is active in AFP-producing hepatoma cells and is silent in AFP-non-producing hepatoma or in fibroblasts; activity of the proximal enhancer is thus cell type-specific. In this respect, it is striking that the main change in chromatin DNAse hypersensitivity, occurring after birth in the liver AFP gene regulatory region, is the suppression of an hypersensitivity site located in the region we defined as the proximal enhancer (Godbout and Tilghman, 1988). This observation strengthens the importance of this region in the developmental regulation of the AFP gene.

In order to determine which transcription factors might control the activity of the AFP proximal enhancer, we dissected this sequence and performed co-transfection assays with transcriptional factor expression vectors. Our data show that the proximal enhancer activity depends on multiple interactions and that c-JUN and C/EBP can be mediators of this activity.

Results

The activity of the AFP proximal enhancer (Ep) depends on multiple interactions

The AFP proximal enhancer (Ep, formerly designated «MN124» fragment; Houart *et al.*, 1990) sequence contains several potential target sites for transcription factors (Fig. 1). To evaluate the importance of these sites, we divided this enhancer into three subfragments (from 5' to 3': Ep1, Ep2 and Ep3) and tested the ability of each of them to stimulate the expression of the TK-CAT reporter gene in hepatoma cells (note that the 5' subfragment Ep1, used in this work, corresponds to the normal sequence deleted of the segment TGGTCTTTGTCCTT, located between positions -171 and -158, covering a hormone response element, and shown to be non-essential for the activity of the enhancer; this deletion was

Abbreviations used in this paper: AFP, α-fetoprotein; bp, base pairs; C/EBP, CAAT/enhancer core binding protein; HNF1, hepatic nuclear factor 1; TK, thymidine kinase (promoter); CAT, chloramphenicol acetyl transferase.

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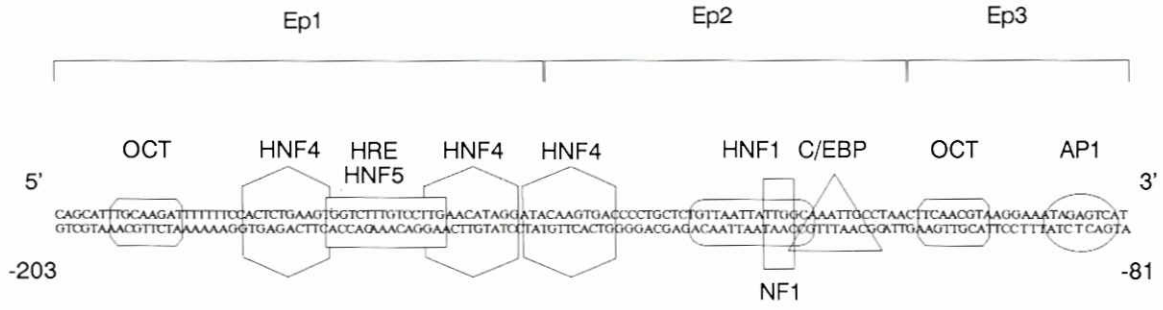


Fig. 1. Sequence of the AFP proximal enhancer. *The following putative binding sites are indicated: OCT=Octamer binding sequence; HNF=Hepatocyte Nuclear Factor binding site; HRE=Hormone Responsive Element (deleted in the Ep1 fragment used in this work); NF1=Nuclear Factor 1 binding site; C/EBP=site for the CAAT/Enhancer core Binding Protein; AP1=JUN/FOS binding site.*

introduced in order to avoid any hormonal interference; see Houart *et al.*, 1990). Each of the three enhancer subfragments was subcloned in the pBL-CAT2 plasmid (Luckow and Schutz, 1987) and the possible enhancer effect of each subfragment on the thymidine kinase promoter was determined (in pBL-CAT2, the bacterial chloramphenicol acetyltransferase gene, CAT, is driven by the herpes virus thymidine kinase promoter, TK, expressed ubiquitously). The different recombinant plasmids were transfected in hepatoma cells. Our results show (Fig. 2) that in human AFP-producing hepatoma cells (HepG2), each of the three fragments is active as an enhancer, at a level similar to that obtained with the entire enhancer sequence. These data imply that the proximal enhancer contains multiple regulatory sites.

The lack of AFP expression in non-producing hepatoma cells (Fa32) seems to be due to the lack of activator factors

The proximal enhancer is not active in the AFP-non-producing hepatoma cells Fa32 (Houart *et al.*, 1990). To study this deficiency, we tested the activity of each of the three enhancer subfragments in these cells. As summarized in Fig. 2, only one of these (Ep2) exhibits some enhancer activity. The Ep2 fragment contains potential binding sites for the transcription factors C/EBP and HNF1, suggesting that at least one of these proteins might be present and active in Fa32 cells, though unable to fully activate the intact proximal enhancer Ep. The lack of enhancer activity of the other subfragments suggests that one or several important factors are absent in these cells.

The c-JUN protein stimulates the proximal enhancer

Among the transcription factors potentially important for the proximal enhancer activity, c-JUN is an attractive candidate. This protein has been widely implicated in various aspects of cell growth and differentiation (see for instance, Ryder *et al.*, 1988; Ryseck *et al.*, 1988; Angel *et al.*, 1989; Brenner *et al.*, 1989; de Groot *et al.*, 1990; Ransone and Verma, 1990; Lamph, 1991). Cell proliferation and AFP expression are correlated, and c-JUN might thus be involved in the control of these two processes. We tested the effect of the c-JUN protein on the AFP proximal enhancer activity by co-transfection of the TK-CAT constructs used above with a c-JUN expression vector. As shown in Fig. 3 (A,B), c-JUN activates the proximal enhancer, at least in AFP-producing cells (HepG2). The data obtained with the enhancer subfragments demonstrate that this activation is medi-

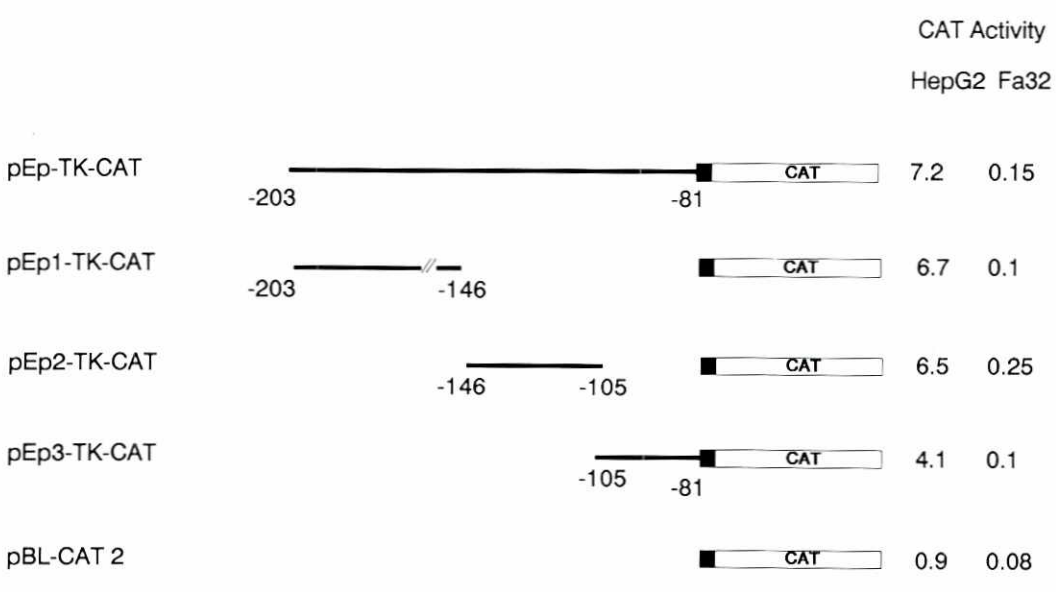
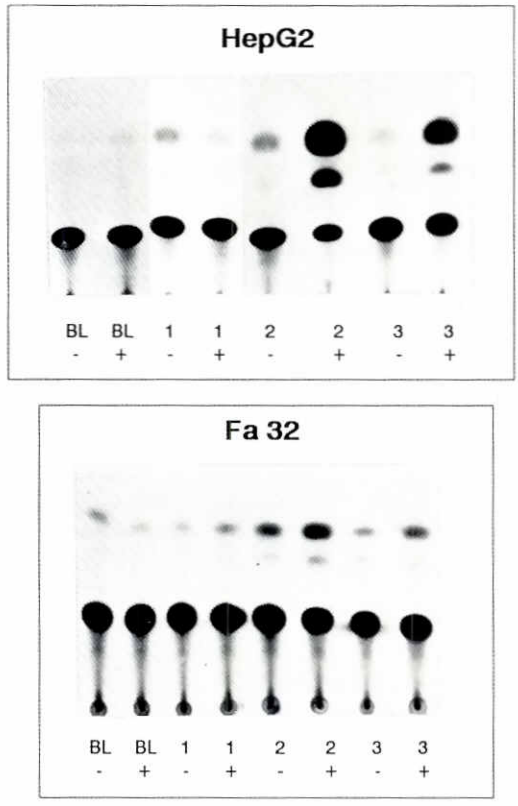


Fig. 2. CAT expression levels of recombinant plasmids in the AFP-producing HepG2 cells and the AFP-non-producing Fa32 cells. *CAT activities are expressed in nmoles of acetylated chloramphenicol/mg of protein. Segments from the AFP proximal enhancer are shown by bars, upstream the TK promoter (black box).*

A

	CAT activity		
	Control	+pRSV-JUN	+pMSV-CEBP
In HepG2 cells:			
pEp-TK-CAT	1.80	4.0	9.50
pEp1-TK-CAT	1.65	1.66	1.70
pEp2-TK-CAT	1.55	1.50	10.7
pEp3-TK-CAT	1.05	6.40	10.0
pBL-CAT2	0.22	0.21	0.55
In Fa32 cells:			
pEp-TK-CAT	0.03	0.10	0.08
pEp1-TK-CAT	0.02	0.02	0.02
pEp2-TK-CAT	0.05	0.04	0.05
pEp3-TK-CAT	0.02	0.15	0.10
pBL-CAT2	0.02	0.02	0.03

B



C

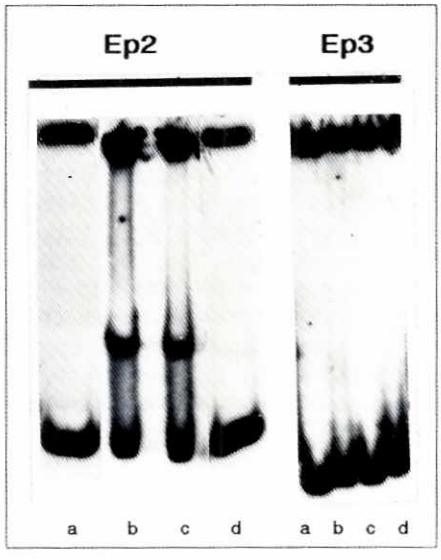


Fig. 3. Effect of c-JUN and C/EBP on the activity of the AFP proximal enhancer. (A) *CAT expression levels directed by recombinant plasmids in the absence (control) or the presence of c-JUN (pRSV-JUN) or C/EBP (pMSV-CEBP) expression vectors in HepG2 and Fa32 cells. CAT activities are expressed in nmoles of acetylated chloramphenicol/mg of protein.* **(B)** *Illustration of CAT assays after transfection of the recombinant plasmids in the absence (-) or the presence (+) of the C/EBP expression vector, in HepG2 (top) or Fa32 (bottom) cells. BL=pBL-CAT2; 1=pEp1-TK-CAT: 2=pEp2-TK-CAT: 3=pEp3-TK-CAT.* **(C)** *Band shift of C/EBP on the Ep2 (left panel) or Ep3 (right panel) subfragments. The shift is clearly visible in the case of Ep2 only. a: probe alone; b: incubation with C/EBP purified protein; c: incubation with C/EBP and 1 mg of pUC unlabeled competitor; d: incubation with C/EBP and 500 ng of unlabeled Ep2 fragment.*

ated by the 3' region (Ep3) of the enhancer sequence. The Ep3 subfragment seems also to be activated by c-JUN in AFP-non-producing cells, but the CAT activity remains low, in comparison with that obtained in HepG2 cells. The Ep3 region contains, in its 3' end, a sequence (AGAGTCA) resembling the typical c-JUN (AP1) binding site (consensus: TGACTCA).

In conclusion, c-JUN is thus able to activate the AFP proximal enhancer and it acts on 3' end of the enhancer, probably via the terminal AGAGTCA sequence.

The C/EBP protein stimulates the proximal enhancer

The C/EBP protein family is involved in the regulation of several genes expressed in the liver, and the C/EBP protein itself is expressed in adult hepatocytes (Landschulz *et al.*, 1988; Birkenmeier *et al.*, 1989; Friedman *et al.*, 1989; Xanthopoulos *et al.*, 1989; Akira *et al.*, 1990; Descombes *et al.*, 1990; Kuo *et al.*, 1990; Poli *et al.*, 1990). Since the AFP gene is silent in these cells, we tested the possible repressor role of C/EBP on the proximal enhancer activity. Co-transfection of the TK-CAT constructs with a C/EBP expression vector was performed. The results, illustrated in Fig. 3 (A,B) demonstrate that C/EBP, surprisingly, exerts a positive effect on the AFP proximal enhancer. C/EBP clearly induces enhancer activity in AFP-producing cells (HepG2) but is inactive, or only moderately active in AFP-non-producing cells (Fa32). Co-transfection experiments with the constructs containing the enhancer subfragments showed (Fig. 3A,B) that in the AFP-producing hepatoma cells (HepG2), C/EBP acts on both the Ep2 and Ep3 regions (7- to 10-fold enhancement). The Ep2 subfragment contains a potential C/EBP binding sequence, unlike Ep3 (Fig. 1), suggesting that the effect of C/EBP observed on the Ep3 sequence in HepG2 is indirect. Gel retardation experiments showed that the C/EBP protein does not bind the Ep3 subfragment, when, as expected, it binds the Ep2 subfragment (Fig. 3C). These data thus indicate that the Ep2 region of the enhancer can be activated by direct binding of the C/EBP

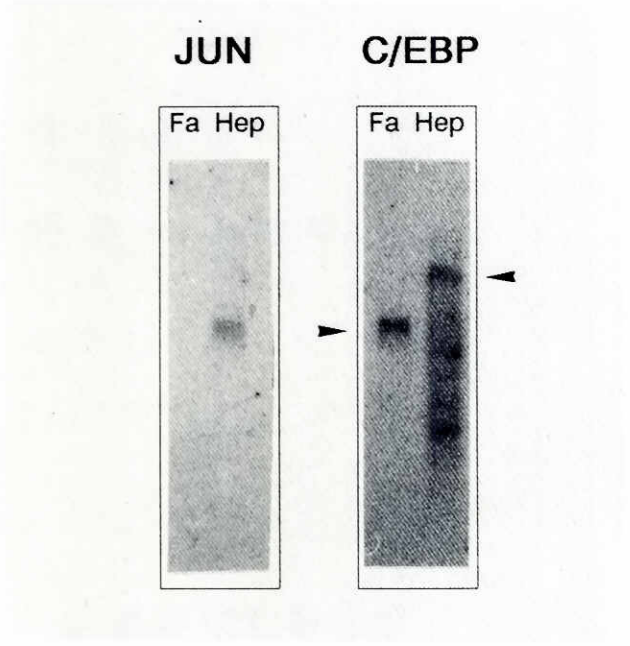


Fig. 4. Northern blot analysis. *Hybridization on total RNA from rat Fa32 (Fa) and human HepG2 (Hep) cells was performed with c-JUN (left panel) and C/EBP (right panel) labeled cDNA. The same filter was used for both hybridizations. The left arrowhead shows the rat C/EBP band (about 2.7 kb); the right arrowhead indicates a human band at 3.5 kb.*

protein, while the Ep3 enhancer activity can be induced indirectly by C/EBP, probably via interaction with another factor.

Expression of the c-JUN and C/EBP mRNAs

The above results suggest that the c-JUN and C/EBP proteins are expressed in the AFP-producing hepatoma cells HepG2. We investigated, by Northern blot analysis, the expression of c-JUN and C/EBP in the two hepatoma lines used in the transfection experiments. As illustrated in Fig. 4 (left panel), c-JUN mRNA was detected in HepG2, at the expected position (about 2.7 kb) but not in Fa32. As also shown in Fig. 4 (right panel), the Fa32 RNA hybridized with the C/EBP probe; since the same filter was hybridized successively with the c-JUN and the C/EBP probes, the lack of c-JUN signal in the Fa32 RNA is thus not due to Fa32 RNA degradation. Fa32 RNA showed a single C/EBP band, at the expected position (about 2.7 kb: Landschulz *et al.*, 1988). On the other hand, several bands were detected in the HepG2 RNA, including a band corresponding to a size (3.5 kb) higher than that of the rat C/EBP mRNA. The extra-bands might be due to expression of an abnormal C/EBP transcript, or to detection of cross-reacting RNAs, encoded by other members of the C/EBP family (Cao *et al.*, 1991; Williams *et al.*, 1991).

We thus conclude that Fa32 cell line lacks c-JUN expression, whereas HepG2 seems to express c-JUN normally. With respect to C/EBP, Fa32 shows the expected mRNA, whereas HepG2 contains several RNA molecular species, related to C/EBP.

Discussion

This study shows that the activity of the AFP proximal enhancer depends on several protein/DNA interactions: we demonstrated that two transcription factors, C/EBP and c-JUN are able to induce this enhancer activity, C/EBP being active on two regions, probably by different mechanisms. Feuerman *et al.* (1989) previously emphasized the role of the HNF1 factor (binding site in the Ep2 region) in tissue-specific expression of the AFP gene. Zhang *et al.* (1991) independently showed that c-JUN stimulates the activity of the AFP promoter and identified a binding site in a region partially overlapping the hormone responsive element deleted in the Ep1 subfragment used in the present study. Their analysis did not exclude the possibility that c-JUN also acts at another site (such as Ep3, as shown in this work). In summary, six important regions that are targets of four transcription factors have been defined in the AFP proximal enhancer: the hormone responsive element (see Houart *et al.*, 1990 and references therein) and a c-JUN binding site in the Ep1 segment (Zhang *et al.*, 1991), a C/EBP binding site and a HNF1 binding site in the Ep2 segment (this study and Feuerman *et al.*, 1989) and undefined sites modulated by C/EBP and c-JUN in the Ep3 segment (this study).

The effect of C/EBP deserves some comments. This factor seems to act on the AFP proximal enhancer in two ways. It binds to a site present in the Ep2 region, and thereby stimulates transcription. But it also activates transcription by acting on the Ep3 segment, to which binding could not demonstrated, and whose sequence does not show any typical C/EBP binding site. This C/EBP effect on Ep3 is thus probably indirect. An interesting possibility is that C/EBP might form heterodimers with another factor possessing a leucine-zipper motif, such as c-JUN. In this respect, it is interesting that c-JUN has been shown to be able to form heterodimers with steroid hormone receptors (for a recent review, see Lamph, 1991) and with members of the activating transcription factor (ATF) family (Hai and Curran, 1991). Furthermore, the resulting heterodimers display altered DNA-binding specificities (Hai and Curran, 1991). It would be interesting to test the possibility that C/EBP and c-JUN are able to form heterodimers and to act, as heterodimers, on the Ep3 segment of the AFP proximal enhancer. An alternative possibility is that C/EBP indirectly activates the Ep3 segment by inducing expression of a gene encoding a transcription factor acting on Ep3.

The observation that c-JUN is able to strongly activate the AFP proximal enhancer seems to be biologically relevant. Indeed, c-JUN expression is closely correlated with cell proliferation, just like AFP expression. Like the AFP gene, the c-JUN gene is silent (or almost

TABLE 1

CAT ACTIVITY

	Control	+pRSV-JUN	+pMSV-CEBP
In HepG2 cells:			
pEp-TK-CAT	1.80	4.0	9.50
pEp1-TK-CAT	1.65	1.66	1.70
pEp2-TK-CAT	1.55	1.50	10.7
pEp3-TK-CAT	1.05	6.40	10.0
pBL-CAT2	0.22	0.21	0.55
In Fa32 cells:			
pEp-TK-CAT	0.03	0.10	0.08
pEp1-TK-CAT	0.02	0.02	0.02
pEp2-TK-CAT	0.05	0.04	0.05
pEp3-TK-CAT	0.02	0.15	0.10
pBL-CAT2	0.02	0.02	0.03

silent) in normal adult hepatocytes and is induced during liver regeneration or carcinogenesis (Ryder and Nathans, 1988; Brenner *et al.*, 1989; Hirai *et al.*, 1989; Sakai *et al.*, 1989; Morello *et al.*, 1990). In this respect, it is striking that the hepatoma line Fa32, which does not express the AFP gene, also lacks significant c-JUN expression. A reasonable hypothesis is that during liver regeneration, AFP gene induction is mediated by c-JUN.

On the other hand, the observation that C/EBP (this work) and HNF1 (Feuerman *et al.*, 1989) are positive regulators of the AFP gene transcription emphasizes the complexity of the developmental regulation of this gene: indeed, these two factors are abundant in the adult hepatocytes, which do not express the AFP gene. Expression of the AFP gene in the hepatocyte lineage is thus dependent on the presence of at least one additional dominant positive regulator (such as c-JUN), or, alternatively, is blocked actively during liver maturation by negative factors (Camper and Tilghman, 1989; Vacher and Tilghman, 1990).

In summary, we have shown i) that the activity of the AFP proximal enhancer (Ep) is dependent on multiple sites, dispersed along the three subregions we designated Ep1, Ep2 and Ep3, and ii) can be stimulated by at least two target sequences (located in Ep2 and Ep3) and two transcription factors, c-JUN and C/EBP. The effect of c-JUN is interesting, taking into account the correlation between cell proliferation, c-JUN expression and AFP expression. We suggest that c-JUN might induce AFP expression during liver regeneration. On the other hand, the stimulatory effect of C/EBP, which is abundant in adult hepatocytes where the AFP gene is silent, suggests that AFP gene expression is dependent on at least one additional dominant positive factor, or is blocked by negative factors associated with hepatocyte maturation.

Materials and Methods

Plasmids and constructions

The plasmids used in this work were derived from the pBL-CAT2 vector (Luckow and Schutz, 1987), which contains the CAT gene driven by the promoter of the herpes simplex virus thymidine kinase (TK). The pEp-TK-CAT plasmid was described earlier under the name «pMN124-TK-CAT» (Houart *et al.*, 1990). The other plasmids were constructed by cloning oligonucleotids (flanked by 5' SalI/XbaI 3' restriction sequences) between the SalI/XbaI sites of the pBL-CAT2 polylinker. Note that the 5' subfragment Ep1 corresponds to the normal 5' sequence of the proximal enhancer (Fig. 1), deleted of the segment TGGTCTTTGTCCTT, located between positions -171 and -158. The pMSV-C/EBP and pRSV-JUN expression vectors contain the C/EBP cDNA, expressed under the control of the MSV 5'LTR, and the c-JUN cDNA expressed under the control of the RSV 5'LTR, respectively (Friedman *et al.*, 1989; Hirai *et al.*, 1990).

Cells and transfections

HepG2 is a human AFP-producing hepatoma cell line (Knowles *et al.*, 1980). Fa32 is a subclone derived from the Reuber H35 rat hepatoma (see Szpirer and Szpirer, 1976 and references therein). Transfections and CAT assays were done as described earlier (Gorman *et al.*, 1982; Molné *et al.*, 1989). Co-transfection experiments were done using 1 mg of CAT reporter plasmid and 10 mg of expression vector per 10^6 cells.

Gel retardation experiments

Gel retardation were performed as previously described (Hennighausen and Lubon 1987).

Northern blot analysis

RNA extracts were done by the guanidium thiocyanate method (Chirgwin *et al.*, 1979). Northern blots were done according to the method described in Sambrook *et al.* (1989).

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