Combinatorial control of positive and negative, upstream and intragenic regulatory DNA domains of the mouse alpha₁-foetoprotein gene

Montse Molné, Corinne Houart, Josiane Szpirer and Claude Szpirer*

Département de Biologie Moléculaire, Université Libre de Bruxelles, Rue des Chevaux, 67, B-1640 Rhode-St-Genèse, Belgium

Received January 27, 1989; Revised and Accepted April 3, 1989

ABSTRACT
Chimeric constructions were isolated, which contained DNA segments from the 5’ flanking region of the mouse AFP gene, placed upstream the bacterial chloramphenicol acetyltransferase gene. Their activity was tested after transfection into different cells. This analysis led to the discovery of two new regulatory elements of the AFP gene. One element is located in the 5’ proximal region flanking the transcriptional start site and acts in a highly cell type-specific manner: it shows an enhancer activity in AFP-producing hepatoma cells, but this proximal enhancer activity is replaced by a strong negative activity in fibroblasts. The second element is located in the intragenic region; it exhibits a negative activity in AFP-producing hepatoma cells where it efficiently antagonizes the proximal enhancer activity; however, this negative effect is overridden by the combined action of the proximal enhancer (identified in this work) and of the distal enhancer identified previously by Godbout and coworkers.

INTRODUCTION
Accumulating evidence indicates that the regulation of gene expression, and in particular of cell-type specific gene expression is mediated by chromatin structure (see for instance 1–5) and by the action of multiple trans-acting regulatory factors on cis-acting DNA-sequence elements (for a recent review, see 6). Chromatin structure is likely to be established by regulatory factors, but might in turn alter the subsequent binding of such factors. The study of several genes also suggests that cell-specific gene expression is ensured by combinatorial controls (7,8); the interplay of these different elements probably allows the cell to reach optimal expression levels in a highly specific manner, controlled both temporally and spatially.

In this respect, the alpha₁-foetoprotein (AFP) gene is an attractive model system. Indeed, the AFP gene is specifically expressed in a limited number of endodermal cell types, during foetal life only (in the visceral endoderm of the yolk sac, the foetal liver, and the gastrointestinal tract); in addition, the AFP gene is expressed in an onco-foetal manner, since it is active in malignant hepatocytes: most hepatomas produce AFP, unlike normal adult hepatocytes (for reviews, see 9–11). Regulation of AFP synthesis occurs at the transcription level (12–14). The mechanisms controlling the cell-specific and onco-foetal expression of the AFP gene are not understood, although some information is available, as summarized below.

Somatic cell genetic experiments have suggested that the AFP gene is subject to trans-acting negative controls, which operate in fibroblasts and extinguish its expression in hepatoma x fibroblast hybrids (15–17). Using cloned mouse AFP gene sequences in transient expression assays, Tilghman and coworkers (18,19) have identified two distinct
Figure 1. General scheme showing the map of the 5′ region of the mouse AFP gene (bottom bar: AFP) and the constructions made by inserting different AFP segments, drawn above the AFP region, upstream the CAT gene of the pSV0-CAT plasmid (26). The pSV0-CAT plasmid and the AFP segments are drawn at different scales; the 1 kb bar refers to the scale of the AFP region and fragments. The name of the constructions is indicated on the left of each inserted segment. These segments were inserted (see Materials and methods) at the HindIII site of pSV0-CAT or between this site and another site located between it and the origin of replication of pBR322 (see Materials and methods; the broken line in the plasmid scheme symbolizes the PvuII site or the Ndel site of the vector sequence). In the AFP gene and its 5′ flanking region, the black bars indicate the exons, the arrow indicates the position of the TATA box and EI and EII shows the position of two distal enhancer elements discovered by Godbout et al. (18). The positions of the pertinent AFP gene restriction sites are shown; this map was partially derived from the map of Kioussis et al. (29) (and verified on our genomic clones), and partially established by us; the indicated sites are: B=BamHI; H=HindIII; N=NlaIV; P=PstI; Pv=PvuII; R=EcoRI; Sm=SmaI; X=XbaI. The limits of the different fragments are indicated: Sm=SmaI site at about -4 kb; N=NlaIV sites at -1 kb and +36 bp; X=XbaI site at -0.8 kb; R=EcoRI site at about +2.5 kb.

regulatory DNA domains in the 5′ flanking region of the mouse AFP gene: a complex enhancer domain, itself divided into three enhancer elements (I,II, III) that lie 2.5, 5.0 and 6.5 kilobases (kb) upstream the transcription initiation site, and a promoter region, containing a TATA box (at 30 bases from the transcription initiation site; 20) and required for specific expression in hepatoma cells (18). Each of these different elements acts in
a tissue-specific manner, though to different extents (18–22). The human and the rat AFP genes are also flanked by an enhancer domain, located at least 2 kb upstream the transcription initiation site (23–25). Finally, an element conferring glucocorticoid repression of AFP gene activity has been identified in the promoter region of the rat AFP gene (25).

In order to analyze the molecular controls which operate on the AFP gene in different cell types, we have examined the capacity of DNA segments from the 5' region of the mouse AFP gene, to drive the expression of a downstream reporter gene, namely the bacterial chloramphenicol acetyltransferase gene (CAT gene; 26). This study allowed us to show first, that the promoter-containing segment also possesses an enhancer regulatory domain ('proximal enhancer') and second, that the AFP intragenic region contains a negative regulatory element which is active in hepatoma cells, where it antagonizes the action of the proximal enhancer element. These two domains, the positive one and the negative one, act in combination with the distal enhancer elements discovered by Tilghman and co-workers (18,19) to control AFP gene expression in hepatoma cells. In addition, our results show that the proximal regulatory domain plays a highly cell-type specific role: its enhancer activity, expressed in AFP-producing hepatoma cells, is replaced by a strong negative activity in fibroblasts.

MATERIALS AND METHODS
Plasmids and Constructions.
Plasmids were constructed and purified using standard protocols (27). The 5' region of the mouse AFP gene was cloned from DNA of the hepatoma cells BWTG3 (28) after EcoRI digestion and insertion in the lambda.gt.wes.lambda B vector. The 8 kb EcoRI fragment overlapping the 5' end of the gene (see 29) was subcloned in pBR325; this fragment runs from the EcoRI site located about 5.5 kb upstream the cap site (this EcoRI site is polymorphic in the mouse and is present in C57B1 mice and in the C57L-derived BWTG3 hepatoma cells; see 30), to the EcoRI site located in the third intron of the gene (29). A 1 kb fragment, containing the promoter region, limited by two NlaIV restriction sites (see Figure 1) was then subcloned at the HindII site of the pUC12 vector; the 3' end of this NlaIV fragment is located between the cap site of the mouse AFP gene and the translation initiation site, at position +36 bp (base pairs) (see the sequence published by Scott and Tilghman, 20); in the recombinant pUC used (pUC-HAFBH; HAF stands for hepatoma AFP), this site was positioned next to the HindII site of the polylinker sequence. Another recombinant pUC was also isolated, where the HAF fragment was oriented in the opposite direction: position +36 bp next the BamHI site of the polylinker (=pUCHAFHB). To test the function of the 1 kb promoter-containing fragment, we excised it from the pUC-HAFBH plasmid by digestion with BamHI and HindIII, and linked it to the bacterial CAT gene, by insertion in the pSV0-CAT plasmid described by Gorman et al. (26) (this plasmid is deleted of the promoter and enhancer sequences present in pSV2-CAT). The original pSV0-CAT plasmid had been modified by converting the NdeI site (located between the origin of replication and the unique HindIII site) into a BglII site; this modified pSV0-CAT was cut with BglII and HindIII and the large fragment was ligated to the 1 kb fragment excised from pUC-HAFBH to yield the pHAF-CAT plasmid. A similar plasmid was constructed where the 1 kb fragment was derived from an AFP genomic clone (lambda MAF-2, a gift from C. Sellem and J. Sala-Trépat) isolated from a mouse plasmacytoma DNA library, 31; this plasmid was called pMAF-CAT (MAF = mouse AFP).
The effect of the enhancer domain identified by Tilghman and co-workers (18, 19), on the 1 kb promoter-containing fragment was tested by placing the enhancer element I upstream this fragment as follows. The pHAP plasmid was cut with Smal (restriction site at about —4 kb from the cap site of the AFP gene) and Xbal (restriction site at about —0.8 kb) and the 3.2 kb Smal-Xbal restriction fragment, containing the enhancer element I was isolated (the Smal site is located to the left of the BamHI site which separates the enhancer elements I and II defined by Godbout et al. (18), and the Smal-Xbal fragment thus also contains the 3' end of enhancer element II; however the enhancer activity of enhancer element II is located upstream the Smal site—see 18— and is thus not present in this fragment). The pHAF-CAT plasmid was cut with Xbal and BamHI, and the 2.45 kb Xbal-BamHI fragment containing 0.8 kb from HAF and the CAT gene was isolated; the pSV2-CAT (26) was cut with PvuII (site located 340 base pairs from the HindIII site, counter-clockwise) and BamHI and the large fragment containing the essential vector sequences was isolated; the three fragments were ligated to yield the pE-HAF-CAT construction, where the 5' upstream region of the AFP gene is regenerated, from the Smal site (—4 kb) to the position +36 bp.

To test the effect of the intragenic 5' end of the AFP gene, the pE-HAF-I-CAT and pHAF-I-CAT were constructed: the pHAP plasmid was digested with Smal and EcoRI and the 6 kb fragment (containing the enhancer element I, the promoter region and about 2.5 kb of intragenic sequence; see Figure 1, and 29) was isolated, blunt-ended and ligated into the pSVO-CAT which had been restricted with HindIII and blunt-ended, to produce pE-HAF-I-CAT. This pE-HAF-I-CAT plasmid was digested with Xbal and BamHI and the 5 kb fragment (which includes the AFP promoter-containing segment, the intragenic segment and the CAT gene) was ligated to the 3 kb Xbal-BamHI fragment isolated from the pHAF-CAT (this fragment contains the vector sequences including the origin of replication), to generate the pHAF-I-CAT plasmid. A deletion removing about 1.5 kb from the 3' end of the intragenic AFP region was generated in pHAF-I-CAT, by partial digestion with PstI (a partial digestion was required, since the pSVO-CAT sequence contains two PstI sites); four PstI sites are located in the intragenic segment used: two in the second intron, and two in the third intron, the last one being close to the 3' end of the intragenic segment (see 29 and Figure 1). The plasmid which had lost the three intragenic AFP PstI-PstI fragments was isolated and re-ligated; this plasmid (pHAF-I_{del}-CAT) thus lacks the sequence located between the first and the fourth PstI sites, i.e. almost all the intragenic sequence downstream the first PstI site, located in the second intron.

The pBL-CAT2 (32) derivatives were constructed as follows. The 1 kb HAF fragment was isolated from the pUC-HAF HB plasmid after digestion with BamHI and HindIII; the pBL-CAT2 plasmid was digested with the same enzymes and the 4.5 kb fragment (i.e. the plasmid deleted from the HindIII-BamHI polylinker) was isolated and ligated to the 1 kb HAF fragment, to generate the pHAF-TK-CAT plasmid. A similar plasmid where the HAF segment is in reverse orientation (pFAH-TK-CAT) was constructed in the same way, starting from the pUC-HAF HB plasmid (instead pUC-HAF HB). The pTK-CAT-HAF and pTK-CAT-FAH plasmids were made by inserting the blunt-ended HAF HB fragment at the Smal site located downstream the CAT gene of the pBL-CAT2 plasmid. All constructions were verified by mapping of restriction fragments.

**Cells and Transfections.**

HepG2 and Hep3B are human hepatoma cell lines (33); BW1 is a mouse hepatoma line (28); these hepatoma lines secrete AFP. The fibroblast line used was the rat RSFTG3 line...
Figure 2. CAT activities directed by the different constructions shown in Fig. 1 and by the control plasmids pSVO-CAT and pSV2-CAT, in the AFP-producing hepatoma cells (human cells: HepG2, Hep3B and mouse cells: BW1) and in rat fibroblasts (RSFTG3); the mouse fibroblasts C11D gave the same results (not shown) as RSFTG3. The DNA transfected were: 0=pSV0-CAT; 2=pSV2-CAT; A=pHAF-CAT; E=pE-HAF-CAT. The spots correspond to: CM=chloramphenicol; 1A=1-acetatechloramphenicol; 3A=3-acetatechloramphenicol; DA=1,3 diacetate chloramphenicol (26).

(a permanent line we isolated from rat embryo skin fibroblasts; 34). All cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum. Transfections were done as described by Graham and Van der Erb (35) and Gorman et al. (26). Cells were inoculated at 2,5 × 10^6 cells per 10 cm dish. The next day, 20 µg of DNA per dish (10 µg of plasmid DNA + 10 µg carrier DNA), precipitated with calcium phosphate, was added to each dish and left on the cells for 8 hours. Cells were harvested 48 hours later, extracts were prepared by 2 cycles of freeze—thawing (36), and were heated for 5 minutes at 65°C (37). CAT assays were performed as described by Gorman (36). After thin-layer chromatography, the spots were cut out and counted. The concentration of protein in the cell extracts was determined by the method of Lowry et al. (38).

RESULTS
The Proximal Region 5’ to the AFP Gene Contains an Enhancer Element.
A 1 kb fragment flanking the 5’ end of the mouse AFP gene was cloned from DNA of the mouse hepatoma cells BWTG3 (fragment HAF). In order to test the biological activity of this fragment, which includes the promoter region, it was introduced upstream the bacterial CAT gene in the pSV0-CAT plasmid, which lacks promoter and enhancer elements (see Figure 1), and the resulting pHAF-CAT plasmid was transfected into hepatoma and fibroblastic cells. The pHAF-CAT plasmid was expressed in the AFP-producing hepatoma cells (human HepG2 and Hep3B cells, mouse BW1 cells), but not in the fibroblasts; these results are illustrated in Figure 2. Expression in HepG2 and Hep3B was very high (expression level higher than that of the positive control plasmid pSV2-CAT: see table 1), as if a strong enhancer-like element was present in the HAF segment. Moreover, the addition of the enhancer element I, located around position −2.5 kb (18, 19; see also Figure 1) only slightly increased the CAT expression level (compare pE-HAF-CAT with pHAF-CAT in Figure 2 and table 1). Removing the 5’ end of the HAF sequence, i.e. the region located upstream the XbaI site (position −0.8 kb) from the 1 kb segment of the pHAF-CAT construction did not alter its activity (pHAF-CAT_{0.8} in Figure 1 and table 2); the active sequence is thus located between position −0.8 kb and the cap site.
TABLE 1: Upstream positive activity and intragenic negative activity.

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>HepG2</th>
<th>BW1</th>
<th>RSFTG3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSVO-CAT</td>
<td>5</td>
<td>1.5</td>
<td>3</td>
</tr>
<tr>
<td>pSV2-CAT</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>pHAF-CAT</td>
<td>140</td>
<td>31</td>
<td>2</td>
</tr>
<tr>
<td>pHAF-CAT_{0,8}</td>
<td>143</td>
<td>32.5</td>
<td>2</td>
</tr>
<tr>
<td>pMAF-CAT</td>
<td>145</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pE-HAF-CAT</td>
<td>175</td>
<td>34</td>
<td>2</td>
</tr>
<tr>
<td>pHAF-I-CAT</td>
<td>15</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>pE-HAF-I-CAT</td>
<td>96</td>
<td>34</td>
<td>1.5</td>
</tr>
<tr>
<td>pHAF-I_{del}-CAT</td>
<td>94</td>
<td>32</td>
<td>2</td>
</tr>
</tbody>
</table>

After thin-layer chromatography, the spots corresponding to the acetylated chloramphenicol forms were cut out and counted. The specific activities were determined for each experiment. The transfected DNA's were: pSVO-CAT (negative control: no promoter or enhancer element), pSV2-CAT (positive control), pHAF-CAT and pHAF_{0,8}-CAT (1 kb or 0.8 kb, respectively, from the 5' upstream region of the hepatoma AFP gene placed upstream the CAT gene; see Figure 1); pMAF-CAT (same as pHAF-CAT, except that the 1 kb fragment was derived from non-hepatoma DNA); pE-HAF-CAT (pHAF-CAT, plus a distal enhancer element placed upstream the HAF segment); pHAF-I-CAT (pHAF-CAT plus a 2.5 kb segment from the 5'end of the intragenic region, placed downstream HAF); pHAF-I_{del}-CAT (pHAF-I-CAT deleted of the PstI restriction fragments); pE-HAF-I-CAT (pHAF-I-CAT plus a distal enhancer element, placed upstream HAF). The absolute values of CAT activity directed by pSV2-CAT (=100%) were: in HepG2: 24.1, in BW1: 25.1, in RSFTG3: 2.1 pmoles acetylated per hour per µg of protein, respectively. The values shown are the means of at least three experiments, made with two independent plasmid preparations; the extreme values corresponding to a given construction in a given cell line did not diverge by more than 15%. ND = not done.

A series of analyses were then done in order to determine whether the positive activity of the HAF fragment fulfills the criteria established for cellular enhancers, i.e., they act on cis-linked promoters in an orientation and position independent manner (see for instance, ref 6). The activity of the HAF segment was thus tested on a heterologous promoter, which exhibits ubiquitous expression. The HAF fragment was inserted in each orientation, upstream and downstream the Herpes virus thymidine kinase (TK) promoter, in the pBL-

TABLE 2: Proximal positive and negative activities in the AFP promoter-containing segment.

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>HepG2</th>
<th>BW1</th>
<th>RSFTG3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSV2-CAT</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>pBL-CAT2</td>
<td>12</td>
<td>49</td>
<td>500</td>
</tr>
<tr>
<td>pHAF-TK-CAT</td>
<td>63</td>
<td>110</td>
<td>32</td>
</tr>
<tr>
<td>pFAH-TK-CAT</td>
<td>59</td>
<td>108</td>
<td>412</td>
</tr>
<tr>
<td>pTK-CAT-HAF</td>
<td>28</td>
<td>58</td>
<td>98</td>
</tr>
<tr>
<td>pTK-CAT-FAH</td>
<td>29</td>
<td>60</td>
<td>501</td>
</tr>
</tbody>
</table>

Legend: see table 1. In this series of experiments, the CAT activities directed by pSV2-CAT (=100%) were: in HepG2: 25.9, in BW1: 25.4, in RSFTG3: 2.1 pmoles acetylated per hour per µg protein, respectively.
CAT2 plasmid, where this promoter drives the CAT gene (32). The resulting constructions were called pHAF-TK-CAT and pFAH-TK-CAT, containing the 1 kb HAF fragment in the 5'-3' or 3'-5' orientation, upstream the TK promoter, and pTK-CAT-HAF and pTK-CAT-FAH, containing this fragment in each orientation downstream the TK promoter and the CAT gene. Table 2 (columns 1 and 2) shows that upon transfection of hepatoma cells, the presence of the HAF segment in the constructions stimulates CAT expression up to 5-fold. This enhancement was observed irrespective of the orientation (in the two hepatoma lines tested), and of the position of the HAF fragment (at least in HepG2 cells). Furthermore, this enhancement was clearly a cis-effect: CAT activity was not enhanced when the TK-CAT construction and the HAF element were carried on distinct plasmids (pBL-CAT2 cotransfected with a pUC plasmid carrying the HAF fragment induced the same CAT activity as pBL-CAT2 alone). These results confirm that the HAF fragment carries a positive element, acting in cis and in an orientation and position independent manner; it will be called proximal enhancer.

The finding of an enhancer activity in the 5' proximal region flanking the AFP gene was unexpected: indeed, previous investigations showed that the 1 kb fragment flanking the 5'end of the AFP gene was unable, alone, to drive active expression of downstream sequences in hepatoma cells (including HepG2): this fragment was described as containing a promoter activity requiring the cooperation of an upstream enhancer element to activate transcription of the downstream gene (18,19,21,23-25). This discordancy could be interpreted in two (non-exclusive) ways: i) our HAF segment, cloned from hepatoma DNA, would carry a mutation generating an enhancer-like element, absent in non-hepatoma AFP DNA (Tilghman and co-workers, for instance, used cloned AFP sequences derived from normal embryo DNA or from plasmacytoma DNA; see 29); ii) the hepatoma HAF segment would lack a negative regulatory element, present in the constructions used by others, and in particular by Tilghman and co-workers, who used an AFP minigene containing intragenic sequences (18, 19). These possibilities were tested as described below.

The AFP 5'Intragenic Region Contains a Negative Regulatory Element, Active in Producing Cells.

On the one hand, the activity of a non-hepatoma 1 kb fragment flanking the 5'end of the AFP gene was tested; this fragment was cloned from plasmacytoma DNA (plasmid pMAF-CAT). As shown in tablel, the non-hepatoma 1 kb fragment MAF was as active as the hepatoma HAF fragment to drive the expression of the CAT gene (plasmids pHAF-CAT and pMAF-CAT). The hepatoma HAF fragment is thus not functionally altered.

On the other hand, a segment of about 2.5 kb from the 5' intragenic region of the AFP gene was placed downstream the HAF fragment (construction pHAF-I-CAT in Figure 1), and the activity of the resulting construction was measured. Table 1 shows that the presence of the intragenic segment abolished CAT expression in the transfected hepatoma cells. This result is in agreement with the data published by Godbout et al., who showed that a similar construction (ZE.5, in ref. 18) is barely transcribed. In addition, to exclude trivial explanations, such as artefactual reading blocking, lack of translation initiation of the CAT sequence, or splicing between a donor site of an AFP intron and the SV40 acceptor site 3' to the CAT gene, the distal enhancer element I was introduced upstream the HAF segment in the pHAF-I-CAT construction, regenerating the complete AFP sequence, from position -4 kb to position +2.5 kb (pE-HAF-I-CAT construction: see Figure 1); it should be noted that this pE-HAF-I-CAT construction is very similar to the pZE.5/I construction of Godbout et al. (18), which was shown to be correctly and actively transcribed in HepG2 cells (18).
As indicated in table 1, the pE-HAF-I-CAT construction recovered a high CAT expression level, similar to that of the pE-HAF-CAT construction which lacks the intragenic region. The negative effect of the intragenic segment is thus relieved by the addition of a distal enhancer, and we conclude that the AFP intragenic region contains a negative regulatory element, active in AFP-producing hepatoma cells and which strongly antagonizes the activity of the proximal enhancer element, but which is itself overridden by the combined action of this proximal element and a distal enhancer. The negative regulatory element could be assigned to the second half of the intragenic region: indeed, removal of the segment located between the first PstI site (position +1.25 kb, in the second intron) and the fourth PstI site (immediately upstream the EcoRI site, in the third intron, at position +2.5 kb) from pHAF-I-CAT restored a high CAT activity (pHAF-I_{del}-CAT: see Figure 1 and Table 1, last line).

The Proximal Enhancer Domain is Cell-Type Specific and Behaves as a Negative Element in Fibroblasts.

To determine whether the enhancer activity exerted by the HAF fragment is cell type-specific, the activity of the pHAF-TK-CAT, pFAH-TK-CAT, pTK-CAT-HAF and pTK-CAT-FAH constructions (described above) was tested in fibroblasts. In these cells, the HAF fragment did not stimulate CAT expression (table 2, last column); moreover, in the case of the pHAF-TK-CAT and pTK-CAT-HAF plasmids (where HAF is in the 5'-3' orientation, upstream and downstream the TK-CAT segment, respectively) the CAT activity was strongly reduced (5 to 15-fold) as compared with that of pBL-CAT2. It thus appears that in fibroblasts, the HAF fragment not only loses its enhancer activity, but, in addition, acquires a negative activity when it is placed in the 5'-3' orientation, either upstream or downstream the linked promoter. This negative effect observed on the TK promoter in the fibroblasts transfected by the pHAF-TK-CAT and pTK-CAT-HAF constructions is highly significant, since the TK promoter of the pBL-CAT2 plasmid is much more active in the fibroblasts used than in the hepatoma cells. The proximal regulatory region of the AFP gene, which behaves positively and in an orientation and position independent manner in AFP-producing hepatoma cells, thus acts negatively in fibroblasts, but in an orientation dependent manner.

DISCUSSION

In this work, two new regulatory elements controlling expression of the AFP gene have been identified. First, an enhancer element was found in the 0.8 kb region upstream the AFP gene; this proximal positive element is active in AFP-producing hepatoma cells and acts in a cell-type specific manner: in fibroblasts, its enhancer activity is replaced by a negative regulatory activity. Second, the 5' end of the intragenic AFP region contains a negative regulatory element, which is active in expressing cells (i.e. in AFP-producing hepatoma cells), and is able, in these cells, to block the activity of the proximal enhancer element. However, this negative control is overridden when the sequence upstream the gene contains not only the proximal regulatory region, but also one of the distal enhancer elements, defined by Godbout et al. (18, 19), and which are located upstream position –2 kb.

The Proximal Enhancer Element and its Cell-Type Specific Action.

The AFP gene is thus characterized by at least four positive elements located upstream the coding region: three distal enhancer elements (I, II, III; see 18,19,22) and one proximal element (this work). Each of these four positive elements individually is able to stimulate
expression of downstream sequences in AFP-producing hepatoma cells. The three distal enhancers exhibit a cell-specific activity to different extents; they have no activity, or a moderate activity in cells not expressing AFP (Hela cells or fibroblasts, 17,18). As regards the proximal element, located in the HAF fragment, its activity is highly cell-specific, as shown by our results on its effects on an heterologous promoter: it stimulates the transcription of a downstream sequence in AFP-producing hepatoma cells, and this positive activity not only disappears in fibroblasts but is there replaced by a negative activity. In these cells, the negative effect of the HAF fragment is also position independent, but it is orientation dependent, whereas in hepatoma cells, its enhancer activity is both position and orientation independent. In at least two other cases (39,40), the effect of negative regulatory elements has been shown to be orientation dependent, when acting on heterologous promoters, as in our experiments. It thus seems that the orientation independent property of enhancers (and of some promoter elements) may not always be shared by negatively-acting ('silencer') elements.

The reason why the AFP proximal enhancer activity is orientation independent (in hepatoma cells), whereas its negative activity is orientation dependent (in fibroblasts) is not known; the putative regulatory factors controlling these two activities might act differently. On the other hand, these two activities might be due either to a common sequence, recognized in expressing hepatoma cells by some positive regulatory factor(s) and in fibroblasts by some negative regulatory factor(s), or to distinct positive and negative elements. The former situation could be illustrated by the recent results of Cereghini et al. (41) who showed that the promoter region of the rat albumin gene contains a sequence motif interacting both with a positive factor (APF) present in liver cells and in differentiated hepatoma cells, and with a putative negative factor (vAPF) present in dedifferentiated hepatoma variants; a related situation has been described by Bamhueter et al.(42) in the case of the β fibrinogen gene promoter, which contains a sequence recognized by a positive nuclear factor (HNF1) present in differenciated hepatocytes and by a variant factor (vHNF) exclusively expressed in dedifferentiated hepatoma cells or in some non-liver tissues. It is tempting to speculate that such variant factors, which might represent alternative spliced products of the homologous positive factors (42) play an important role in the control of the non-hepatic phenotype. The latter situation (distinct positive and negative elements) is illustrated by several genes flanked by a complex regulatory region containing interspersed positive and negative elements, where the negative elements are active only in non-expressing cells, where they probably bind repressor proteins (see for instance the rat insulin gene, 43,44; the human retinol-binding protein, 7, or the immunoglobulin heavy chain genes, 45).

The variant nuclear factors and the repressor molecules are probably responsible for the extinction phenomenon observed in somatic cell hybrids, and in particular for the extinction of AFP in hepatoma×fibroblasts hybrids. It has already been suggested by Widen and Papaconstantinou (17) that the 1 kb promoter-containing region upstream the AFP gene might play a critical role in the extinction phenomenon: these authors showed that the AFP promoter activity is dramatically lowered in fibroblasts and in hepatoma×fibroblast hybrids. In our case, we have shown that the stimulating activity of the 1 kb proximal control region on a heterologous promoter in hepatoma cells is replaced by a negative activity in fibroblasts. Both observations could be explained by the action of the same negative regulatory factor(s), present in fibroblasts. However, our result showing that the addition of the HAF segment upstream the thymidine kinase promoter, active in fibroblasts,
inhibits this promoter activity indicates that the putative fibroblast negative factor(s) is (are) acting via binding to a DNA sequence located in the AFP proximal control region. The Intragenic Negative Activity.

Our finding that the proximal control region upstream the mouse AFP gene contains an enhancer activity is not inconsistent with the fact that Godbout et al. (18) did not detect any proximal enhancer activity. Indeed, these authors used AFP minigene constructions, containing intragenic AFP sequences, including the 5' end of the gene, and we have now shown that this intragenic region (and more precisely, the segment located between positions +1.5 kb, in the second intron, and +2.5 kb, in the third intron) contains a negative regulatory element, active in AFP-expressing cells, and which cancels the activity of the proximal enhancer element. This negative effect of the intragenic element can be overridden by the contribution of a distal enhancer element. This observation suggests that AFP gene transcription is controlled by a three-dimensional structure assembling several non contiguous DNA segments, via the binding of interacting proteins (see ref. 46). The behaviour of the AFP intragenic negative element differs from that we observed in the case of the proximal upstream region (HAF fragment), or with the examples mentioned above (rat insulin gene, 43, 44; human retinol-binding protein, 7, or immunoglobulin heavy chain genes, 45) where the negative element acts negatively only in non-expressing cells. The existence of negative elements active in expressing cells has been suggested in the case of the AFP gene, in the upstream region of the gene (rat AFP gene, at position —3.5 kb, see 23; mouse AFP gene, in the enhancer element I; see 19); such a negative element has also been described in the case of a class I MHC gene (39). This type of negative elements active in expressing cells might be more general than suspected today and might play an important role in the control of gene expression.

In conclusion, our work emphasizes the multiplicity of the controls regulating the activity of the AFP gene. Several DNA domains, acting positively and negatively, and located upstream and downstream the cap site, combine their effects to direct the cell-type specific expression of this gene.

ACKNOWLEDGEMENTS

We thank R. Thomas for thoughtful reading of the manuscript and suggestions, M. Rivière and P. Drèze for excellent technical assistance, L. Piérad and D. Christophe for advice in the initial phase of this study, D. Glansdorf for help in the late stage of the work and K. Opdecamp for active discussions. We are also grateful to G. Schütz and B. Luckow for the gift of pBL-CAT2. This work was supported by the CGER-ASLK, the Fund for Joint Basic Research (FRFC, Belgium), and the Association contre le Cancer (Belgium). M.M. was supported in part by a fellowship from CIRIT (Catalunya Generalitat, Barcelona); C.H. is an IRSIA grantee; C.S. is senior research associate of the National Fund for Scientific Research (FNRS, Belgium).

*To whom correspondence should be addressed

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