MOLECULAR AND CELLULAR BIOLOGY, Dec. 1991, p. 5885–5893
0270-7306/91/125885-09$02.00/0

Vol. 11, No. 12

# A Position-Dependent Silencer Plays a Major Role in Repressing α-Fetoprotein Expression in Human Hepatoma

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Received 15 May 1991/Accepted 6 September 1991

**A large percentage of human hepatomas produce α-fetoprotein (AFP), but the levels of AFP expression vary greatly among hepatomas. To understand the molecular basis for this variation, we analyzed transcriptional regulatory activities associated with the 5′-flanking region of the AFP gene in two human hepatoma cell lines, HuH-7 and huH-1/cl-2, which produce a high and a low level of AFP, respectively. We found that the low level of AFP production in huH-1/cl-2 is due to the action of at least two silencer regions located between the enhancer and the promoter of the AFP gene. In contrast, no silencer activity is expressed in HuH-7. We identified 5′-CTTCATAACTAATACTT-3′ to be a core sequence responsible for the negative regulatory activity. This sequence is repeated four times in a strong, distal silencer region, Sd, whereas one copy is present in a weak, proximal silencer region, Sp. The silencer reduces transcriptional initiation by blocking enhancer activation of the AFP promoter in a position-dependent manner. The silencer functions in the presence of positive transcription factors and may play a key role in developmental repression as well as variable expression of the AFP gene in hepatomas.**

The α-fetoprotein (AFP) gene is a developmentally regulated gene whose activity decreases rapidly after birth and becomes hardly detectable in adult life. However, the AFP gene is often reactivated to a high level in hepatocellular carcinoma (HCC) and teratocarcinomas (1, 4, 36). In contrast, the expression of the albumin gene, which is evolutionarily related to the AFP gene, steadily increases during normal development and stays high throughout adult life.

Elevated levels of AFP have been observed in more than 70% of HCC patients, making AFP as a reliable diagnostic and prognostic marker of HCC. The AFP levels among AFP-positive patients, however, vary widely. Analysis of mechanisms underlying the variation of AFP production in HCC is of importance from clinical as well as basic biological standpoints.

HuH-7 and huH-1/cl-2 are two human hepatoma cell lines that exhibit various liver-specific functions (23–25). HuH-7 produces a large amount of AFP and a moderate level of albumin. huH-1/cl-2, on the other hand, produces only a detectable amount of AFP but a high level of albumin. These cell lines provide useful model systems for the study of the molecular basis for different levels of expression of the AFP and albumin genes in hepatoma.

We have shown previously that in HuH-7 cells, AFP gene expression is regulated positively by the enhancer, promoter, and glucocorticoid-responsive element (27, 32, 39). We have not so far detected DNA elements that suppress AFP gene transcription in this cell line. In this study, we analyzed mechanisms responsible for a low level of AFP gene expression in huH-1/cl-2. Two possibilities were considered: (i) the reduced activity of positive regulatory elements and (ii) the activation of negative control elements. We present evidence that the latter mechanism is largely responsible for the low level of AFP production in huH-1/cl-2.

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## MATERIALS AND METHODS

**Cell cultures and transfection analysis.** The HuH-7 human hepatoma cell line was cultured in a chemically defined medium ISE-RPMI, which contains ethanolamine (30 μg/ml) in IS-RPMI (23, 24). huH-1/cl-2, a clone isolated from the parental huH-1 human hepatoma cell line (17), was cultured in ISE-RPMI with 1% fetal calf serum.

Transfection was performed by the calcium phosphate precipitation method as described previously (27). The transfected cells were lysed by several cycles of freezing and thawing and centrifuged at 15,000 rpm for 5 min. The supernatant was heated at 60°C for 10 min (34) and analyzed for chloramphenicol acetyltransferase (CAT) activity according to Gorman et al. (15). This heat treatment is essential for the detection of CAT activity in huH-1/cl-2 cells. Similar heat treatment of extracts resulted in a 3.5-fold increase in HuH-7 cell CAT activity. The specific CAT activities of pSV2-CAT (15) in various cell lines are shown in Table 1.

**Construction of CAT fusion genes.** CAT fusion genes were constructed by linking the CAT gene to AFP or albumin 5′-flanking sequences obtained by restriction enzyme digestion or polymerase chain reaction and inserting them into the *Hin*dIII site of pBR-CAT (39).

Internal deletion mutants were constructed by deleting various lengths of DNA downstream of −2.9 kb (*Bgl*II) in pAF5.1-CAT, which contains 5.1 kb of the AFP 5′-flanking sequence (39).

pSV1.6-CAT, which contains one 72-bp element of the simian virus (SV40) early promoter, was constructed by removing one 72-bp element from pSV2-CAT by treatment with *Sph*I followed by recircularization through *Bgl*II linkers.

The structures of pSV1′-CAT, pSVAF2.4-CAT, and pSVAF0.4-CAT were described previously (39). To construct pSVAF0.3-CAT, a 322-bp AFP enhancer domain A

TABLE 1. Expression of CAT activity from various AFP-CAT fusion genes

| Cell line | CAT activity | | | | | |
|---|---|---|---|---|---|---|
| | pSV2-CAT | | pSVAF-2.4-CAT % | pSVAF-0.3-CAT % | pSVAF-0.4-CAT % | pSV1'-CAT % |
| | pmol/h/μg of protein | % | | | | |
| HuH-7 | 731 | 100 | 62 (44) | 22 (16) | 5.9 (4) | 1.4 (1) |
| huH-1/cl-2 | 46 | 100 | 68 (38) | 25 (14) | 7.2 (4) | 1.8 (1) |
| HeLa | 32 | 100 | 3.5 (1.7) | 3.4 (1.6) | 3.2 (1.5) | 2.1 (1) |

[a] Values in parentheses indicate fold stimulation over pSV1'-CAT activity.

fragment (−4.0 to −3.7 kb) was inserted into the *Bgl*II site of pSV1'-CAT.

**Preparation of nuclear extracts.** Nuclear extracts from HuH-7 and huH-1/cl-2 cells were prepared according to Dignam et al. (11) and Shapiro et al. (33), with some modifications. Cells were treated with hypotonic buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid [HEPES; pH 7.9]), 10 mM KCl, 0.1 mM EDTA, 0.75 mM spermidine, 0.15 mM spermine, 0.5 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 300 mU of aprotinin per ml) for 10 min on ice and homogenized in an all-glass Dounce homogenizer, using the A (tight) pestle. Nuclei were collected by centrifugation for 30 s at 10,000 rpm and lysed in lysis buffer (20 mM HEPES [pH 7.9], 25% glycerol, 0.5 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 300 mU of aprotinin per ml) for 30 min at 4°C. Nuclear proteins were precipitated by the addition of $(NH_4)_2SO_4$ (40%), collected by centrifugation, and dialyzed against 20 mM HEPES (pH 7.9)–20% glycerol–0.1 M KCl–0.2 mM EDTA–0.5 mM DTT–0.5 mM PMSF. Insoluble material was removed by centrifugation, and the supernatant was stored in small aliquots at −70°C. Protein concentration of the nuclear extracts was determined by the Bio-Rad protein assay kit.

**Gel mobility shift assays.** A 15-bp synthetic oligonucleotide, 5'-TGATTAATAATTACA-3', corresponding to the AT-rich element in the human AFP enhancer (32), and a 22-bp NF-1/CTF binding site, 5'-ATTTTGGCTTGAAGCC AATATG-3' (19), were used as probes. Nuclear extracts were incubated with $3 \times 10^3$ cpm of $^{32}P$-labeled DNA probes for 20 min at 24°C in the presence of 10 mM Tris-HCl (pH 7.5), 45 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 5 μg of poly(dI-dC) · poly(dI-dC), 1 μg of denatured salmon sperm DNA, and 8% glycerol. The reaction mixture was electrophoresed in a 4% polyacrylamide gel containing 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA (32) with circulating buffer at 4°C. The gel was dried and autoradiographed at −70°C. The amount of DNA-protein complexes was quantified by scintillation counting of the radioactivity associated with the retarded band.

**Primer extension analysis.** Primer extension analysis of CAT transcripts was conducted according to Bodner and Karin (3), with minor modifications. Total RNA (100 μg) was incubated with a primer corresponding to the sequence from +26 to +49 of the CAT gene, 5'-CAACGGTGGTATATCCA GTG-3', labeled with $^{32}P$ ($2 \times 10^4$ cpm), in 10 μl of 10 mM Tris-HCl (pH 7.9)–1 mM EDTA–0.25 M KCl at 50°C; 24 μl of 10 mM Tris-HCl (pH 8.7) containing 10 mM $MgCl_2$, 5 mM DTT, 100 μg of actinomycin D per ml, 0.4 mM each of four deoxynucleoside triphosphates, and 1.0 U of reverse transcriptase (Molecular Genetics) was added, and the reaction mixture was further incubated for 1 h at 42°C. The transcription products were isolated by phenol extraction, precipitated with ethanol, dissolved in 80% formamide with 0.01% xylene cyanol and 0.01% blomophenol blue, and electrophoresed on a 6% polyacrylamide gel containing 7 M urea.

## RESULTS

**Different levels of production of AFP and albumin in HuH-7 and huH-1/cl-2 human hepatoma cell lines.** The amounts of AFP and albumin secreted into media by HuH-7 and huH-1/cl-2 over a 10-day period of growth are shown in Fig. 1A. The AFP secretion by HuH-7 was 75- to 100-fold larger than that by huH-1/cl-2 (150 to 200 versus 2 ng/24 h/$10^4$ cells). Consistent with these results, AFP mRNA was readily detected in HuH-7 but not in huH-1/cl-2 by Northern (RNA) blot analysis (Fig. 1B). Albumin expression, on the other

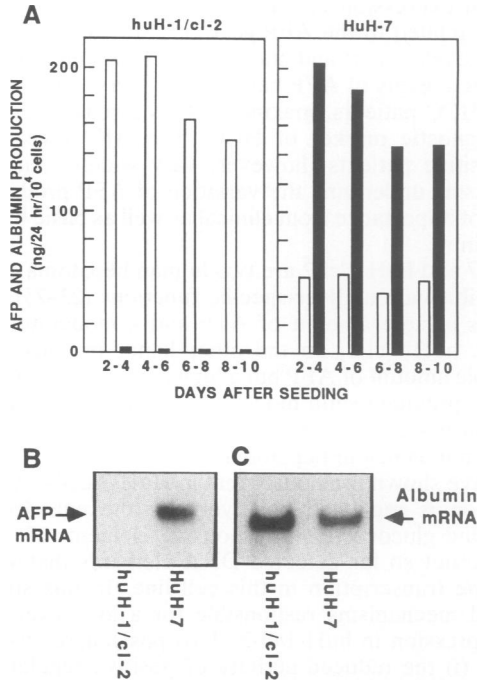


FIG. 1. Production of AFP and albumin in HuH-7 and huH-1/cl-2 human hepatoma cell lines. (A) AFP and albumin secretion. The amounts of AFP and albumin secreted into the medium by huH-1/cl-2 and HuH-7 were determined by enzyme immunoassay (25) over a 10-day period. Open bars, albumin; solid bars, AFP. (B and C) AFP and albumin mRNAs. Northern blot analysis of AFP and albumin mRNAs was performed by using $^{32}P$-labeled AFP cDNA (pHAF-2 [21]) and albumin cDNA (phalb-7 [37]) as probes. (B) 2.2-kb AFP mRNA; (C) 2.2-kb albumin mRNA.

hand, was about three times higher in huH-1/cl-2 than in HuH-7 in terms of both albumin secretion (Fig. 1A) and albumin mRNA level (Fig. 1C).

**Expression of CAT gene driven by the AFP and albumin 5′-flanking DNA in HuH-7 and huH-1/cl-2.** To determine whether the differences in AFP and albumin production in HuH-7 and huH-1/cl-2 cells are due to differences in transcriptional regulatory activities of the AFP and albumin 5′-flanking regions, we transfected these cells with plasmids containing the CAT gene to which various lengths of AFP or albumin 5′-flanking sequences are linked (Fig. 2A). The 5.1-kb AFP 5′-flanking DNA which contains the full AFP enhancer region (−4.9/−2.9 kb) supported a high level of CAT expression in HuH-7 (Fig. 2B, lane 3) but only a low level of expression in huH-1/cl-2 (Fig. 2B, lane 8). CAT expression supported by the 1.0-kb AFP promoter region was also greater in HuH-7 than in huH-1/cl-2 (Fig. 2B, lanes 4 and 9). In contrast, albumin 5′-flanking sequences, either 3 kb (pALB3.0-CAT) or 0.7 kb (pALB0.7-CAT), supported about threefold-higher CAT activity in huH-1/cl-2 than in HuH-7 (Fig. 1B, lanes 1, 2, 6, and 7). Thus, there is a correlation between transcriptional activities of the AFP or albumin 5′-flanking sequences and the levels of AFP or albumin production in HuH-7 and huH-1/cl-2, respectively.

Comparison of CAT activities supported by 5.1 kb (containing the AFP enhancer) and 1.0 kb (without the AFP enhancer) of the AFP 5′-flanking sequence showed that the AFP enhancer stimulated the AFP promoter activity 25-fold in HuH-7 (Fig. 2B, lanes 3 and 4) but only 2-fold in huH-1/cl-2 (Fig. 2B, lanes 8 and 9). In contrast, there was little difference between these cell lines in the level of stimulation of the albumin promoter by the albumin enhancer (3-fold) (Fig. 2B, lanes 1, 2, 6, and 7). These results indicate that in huH-1/cl-2, the AFP enhancer does not stimulate the AFP promoter as much as in HuH-7.

**Analysis of AFP promoter and enhancer activities in HuH-7 and huH-1/cl-2.** To show that enhancer activation of the AFP promoter is selectively inhibited in huH-1/cl-2 as suggested above, it is important to establish that both the AFP enhancer and the promoter are active in this cell line. To analyze the functionality of the AFP promoter, we tested the effect of dexamethasone on the AFP promoter activity. This test is based on the observation that dexamethasone stimulates the AFP promoter only when it is functional (27). We found that dexamethasone stimulated CAT activity supported by the 1.0-kb AFP promoter 9- and 12-fold in huH-1/cl-2 and HuH-7, respectively (Fig. 2C). This result indicates that the AFP promoter is functional in huH-1/cl-2.

Next we determined the AFP enhancer activity in HuH-7 and huH-1/cl-2. The 2.4-kb full enhancer region (−5.3/−2.9 kb) and two subregions, 0.3-kb domain A (−4.0/−3.7 kb) (26) and 0.4-kb domain B (−3.7/−3.3 kb) (32, 39) (Fig. 2D), were individually tested in conjunction with the SV40 promoter. All of these enhancer regions stimulated the SV40 promoter activity to essentially the same degrees in HuH-7 and huH-1/cl-2 (Table 1). This finding indicates that the AFP enhancer elements are as active in huH-1/cl-2 as in HuH-7.

The results presented above suggest that positive transcription factors regulating the AFP enhancer and promoter are not limiting in huH-1/cl-2. To confirm this interpretation, we performed gel mobility shift assays to analyze the levels of two transcription factors, AFP1 (also called HNF1/LF-B1), which binds to AT-rich sequences in the AFP enhancer and promoter (7–10, 13, 14, 20, 32), and NF-1/CTF, which interacts with the AFP promoter (13). Extracts from both HuH-7 and huH-1/cl-2 formed retarded bands with synthetic

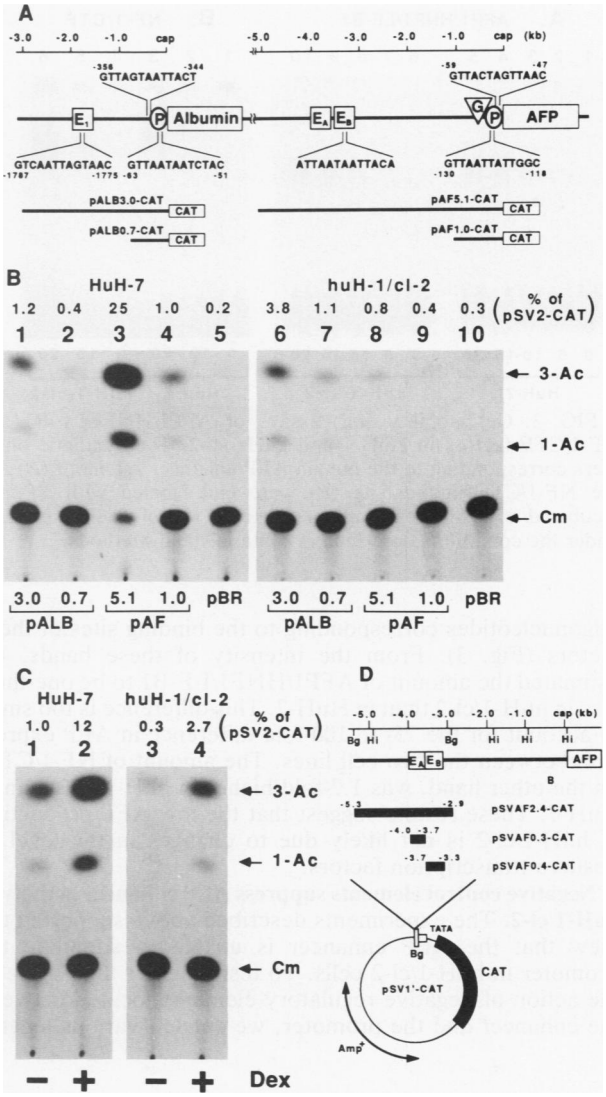


FIG. 2. CAT fusion genes containing AFP or albumin 5′-flanking sequences and their expression in HuH-7 and huH-1/cl-2 cells. (A) CAT constructs containing various lengths of AFP or albumin 5′-flanking sequences. AFP and albumin 5′-flanking sequences linked to the CAT gene are drawn under the albumin-AFP locus. Positions of the enhancers ($E_1$, $E_A$, and $E_B$), promoters (P), glucocorticoid-responsive element (G), and AT-rich elements are also indicated. (B) CAT activities expressed in HuH-7 and huH-1/cl-2 cells transfected with the CAT fusion genes shown in panel A. Transfection and analysis of CAT activity were conducted as described in Materials and Methods. The amounts of extract and incubation times were 25 μg and 20 min for HuH-7 (lanes 1 to 5) and 100 μg and 180 min for huH-1/cl-2 (lanes 6 to 10). CAT activities as percentages of pSV2-CAT activity are shown above the autoradiograms. Cm, chloramphenicol; 1-Ac, 1-acetate chloramphenicol; 3-Ac, 3-acetate chloramphenicol. (C) Effects of dexamethasone on CAT activities in HuH-7 and huH-1/cl-2 cells. HuH-7 and huH-1/cl-2 cells were transfected with pAF1.0-CAT, incubated with or without of $3 \times 10^{-6}$ M dexamethasone for 2 days, and analyzed for CAT activity. The amounts of extract and the incubation times were 25 μg and 20 min for HuH-7 (lanes 1 and 2) and 100 μg and 180 min for huH-1/cl-2 (lanes 3 and 4) Cm, 1-Ac, and 3-Ac are as defined for panel B. + and − indicate the presence and absence of dexamethasone, respectively. (D) Diagram of the AFP enhancer/SV40 early promoter/CAT fusion genes. pSVAF2.4-CAT contains the entire AFP enhancer region. pSVAF0.3-CAT and pSVAF0.4-CAT contain domains A and B, respectively. Hi, *Hin*dIII; Bg, *Bgl*II.

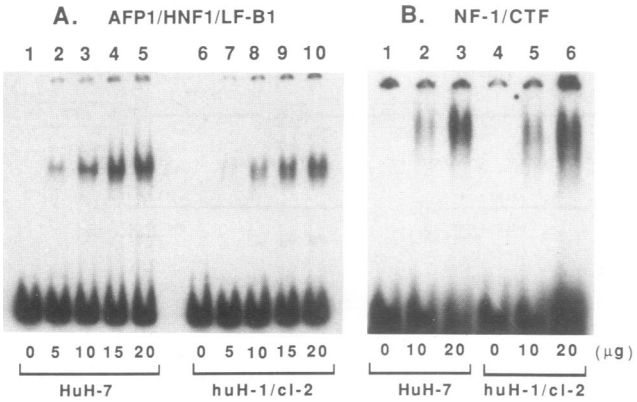


FIG. 3. Gel mobility shift assays of AFP1/HNF1/LF-B1 and NF-1/CTF factors in HuH-7 and huH-1/cl-2. The synthetic oligomers corresponding to the human AFP enhancer AT motif (A) and the NF-1/CTF-binding site (B) were end labeled with $^{32}P$ and incubated with different amounts (5 to 20 μg) of nuclear extracts under the conditions described in Materials and Methods.

oligonucleotides corresponding to the binding sites of these factors (Fig. 3). From the intensity of these bands, we estimated the amount of AFP1/HNF1/LF-B1 to be one-third less in huH-1/cl-2 than in HuH-7. This difference is too small to account for the 75- to 100-fold difference in AFP expression between the two cell lines. The amount of NF-1/CTF, on the other hand, was 1.7-fold higher in huH-1/cl-2 than in HuH-7. These results suggest that the low AFP production in huH-1/cl-2 is not likely due to changes in the level of positive transcription factors.

**Negative control elements suppress AFP enhancer activity in huH-1/cl-2.** The experiments described above supported the view that the AFP enhancer is unable to stimulate the promoter in huH-1/cl-2 cells. To test whether this is due to the action of negative regulatory elements located between the enhancer and the promoter, we deleted various lengths of DNA downstream of the enhancer (at −2.9 kb) of the 5.1-kb AFP 5′-flanking sequence in pAF5.1-CAT (Fig. 4). These deletions had little effect on CAT expression in HuH-7 (Fig. 4). In huH-1/cl-2, on the other hand, CAT expression changed with several deletions. In particular, significant increases were observed with deletions from −2.9 kb to −951 bp ([Δ2.0] in Fig. 4) (3.6-fold) and from −2.9 kb to −169 bp ([Δ2.7] in Fig. 4) (10-fold). These results suggest that at least two negative control regions exist between −1822 and −169 bp, one from −1822 to −951 bp (distal silencer [Sd]) and the other from −402 to −169 bp (proximal silencer [Sp]). In addition, we observed a small but consistent increase in CAT activity associated with the deletion from −951 to −402 ([Δ2.5] in Fig. 4), which may suggest the presence of a third, weak negative element in this region.

The 875-bp Sd region was analyzed for transcription-suppressive activity by inserting it back to pAF5.1[Δ2.7]-CAT, in which a 2.7-kb sequence from −2.9 kb to −169 bp had been deleted. The resultant construct ([Δ2.7]+875) showed a fivefold-lower CAT activity than the parental plasmid ([Δ2.7]) in huH-1/cl-2 (Fig. 4). To further delimit the silencer activity, the 875-bp DNA was divided into two fragments, a 409-bp 5′ fragment (from −1822 to −1414) and a 389-bp 3′ fragment (from −1336 to −948), and tested separately for suppressive activity. The 409-bp 5′ fragment ([Δ2.7]+409) suppressed CAT activity strongly, whereas the 3′ fragment ([Δ2.7]+389) did so only weakly (Fig. 4). This finding indicates that the major silencer activity is contained in the 409-bp 5′ fragment. In the absence of the enhancer (0.2+409), the 5′ fragment weakly stimulated AFP promoter activity (Fig. 4). These results indicate that the action of Sd is to interfere with AFP enhancer activity without affecting AFP promoter activity.

In the absence of the entire suppressor region ([Δ2.7]), the CAT activity expressed in huH-1/cl-2 is fourfold lower than that in HuH-7 (25.9 versus 116). This finding suggests that a mechanism exists in huH-1/cl-2 to suppress the intrinsic AFP promoter activity, although this accounts only partially for

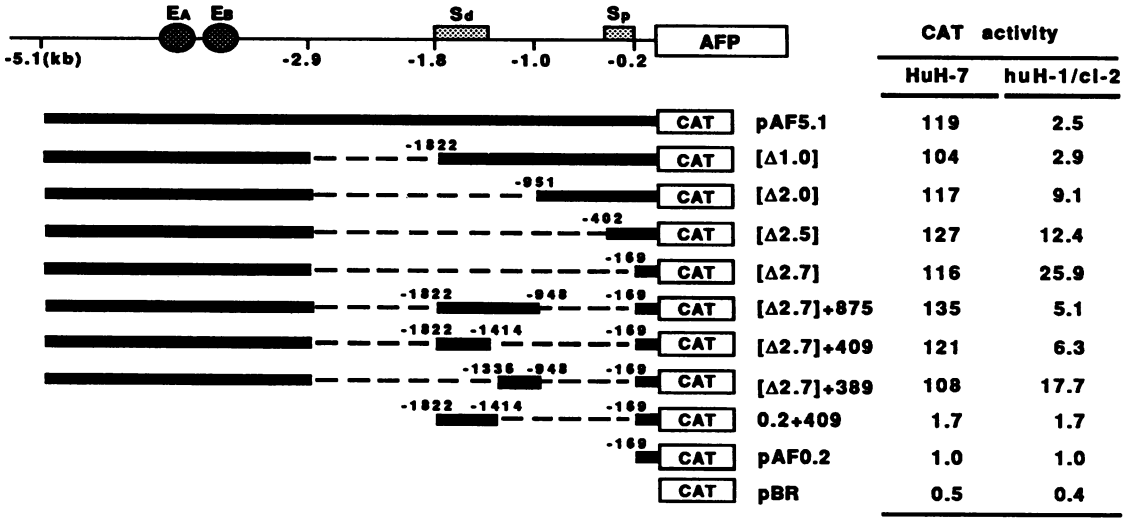


FIG. 4. Detection of silencer activities by deletion analysis. Various lengths of DNA downstream of the AFP enhancer (starting at −2.9 kb) were deleted from the 5.1-kb AFP 5′-flanking sequence in pAF5.1-CAT. The precise endpoints of deletion are indicated in base pairs from the AFP cap site. The closed bars indicate AFP 5′-flanking sequences, and the dotted lines indicate deleted sequences. The CAT activities shown on the right are expressed relative to that of pAF0.2(Bg)-CAT (pAF0.2), which contains the 169-bp AFP promoter region. The results are averages of three independent transfection experiments.

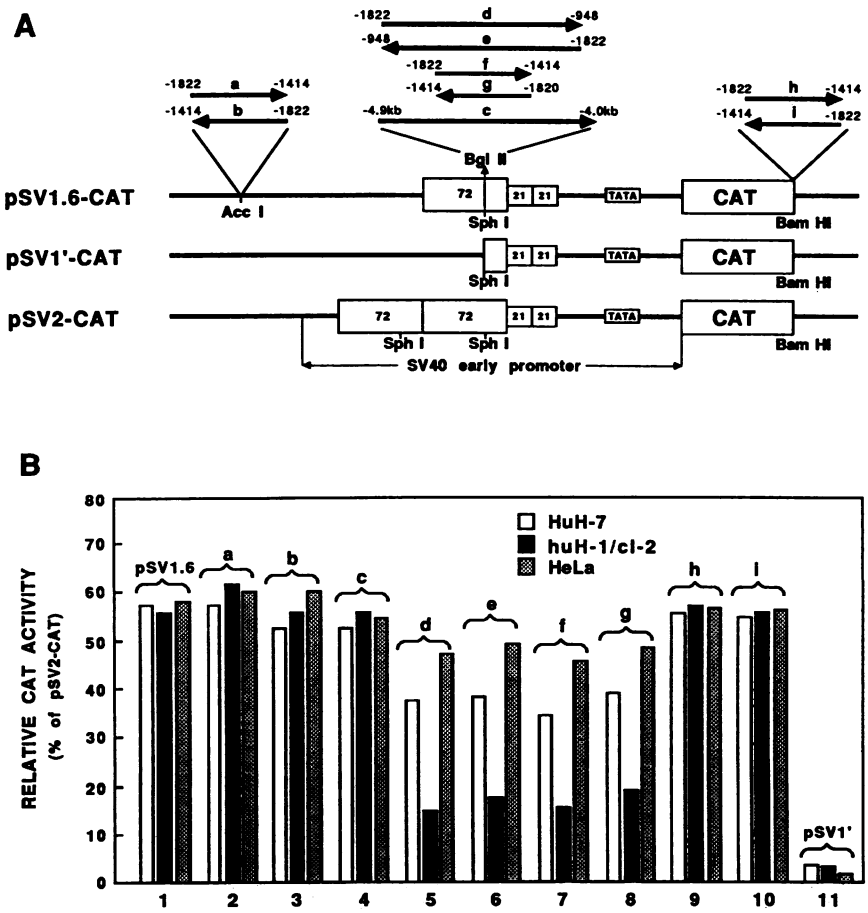


FIG. 5. Position-dependent suppression of SV40 enhancer activity by the Sd silencer region. (A) The 875-bp Sd region (from −1822 to −948 bp) was inserted to the *Bgl*II site of pSV1.6-CAT in normal (d) and reverse (e) orientations. The 409-bp 5′ fragment (from −1822 to −1414 bp) was inserted to the *Acc*I (a and b), *Bgl*II (f and g), or *Bam*HI (h and i) site in normal and reverse orientations. A nonfunctional 924-bp fragment (from −4.9 to −4.0 kb) was inserted to the *Bgl*II site (c) as a control. (B) CAT activities expressed in HuH-7, huH-1/cl-2, and HeLa cells after transfection with CAT fusion genes shown in panel A. The results are averages of three independent transfection experiments.

the large difference in AFP production between HuH-7 and huH-1/cl-2.

**The Sd element suppresses SV40 enhancer activity in a position-dependent manner.** The effect of the Sd region on a heterologous enhancer was examined by inserting the 875-bp Sd region in the SV40 enhancer of pSV1.6-CAT (*Sph*I site converted to *Bgl*II site) in normal and reverse orientations (Fig. 5A, d and e). The resultant constructs expressed much lower CAT activity in huH-1/cl-2 than did the parental plasmid, indicating that Sd can suppress the heterologous promoter in an orientation-independent manner (Fig. 5B, columns 5 and 6). Suppression of CAT activity was also observed in HuH-7 and HeLa cells although to much lesser extents (Fig. 5B, columns 5 and 6). This finding suggests that the Sd activity is not strictly cell type specific. To show that the observed effects are not due to the disruption of the SV40 enhancer, we tested the effect of an insertion of a 924-bp DNA from −4.9 to −4.0 kb of the AFP gene with no known regulatory activity at the same site of pSV1.6-CAT (Fig. 5A, c). No significant changes in CAT expression was observed in all cell lines tested (Fig. 5B, column 4).

The orientation-independent suppression was also observed with the 409-bp Sd fragment inserted in the SV40 enhancer (Fig. 5B, column 7 and 8). However, when it was inserted upstream of the SV40 enhancer (Fig. 5A, a and b) or downstream of the CAT gene (Fig. 5A, h and i), no suppressive effect was observed in either orientation in any of the cell lines tested (Fig. 5B, columns 2, 3, 9, and 10). These results show that the effect of Sd is orientation independent but position dependent.

**Primer extension analysis of CAT transcripts in transfected huH-1/cl-2 cells.** To determine whether Sd suppresses transcriptional initiation or changes the transcription start site, we performed primer extension analysis of CAT transcripts in huH-1/cl-2 transfected with pSV1.6-CAT with or without the 409-bp Sd region inserted in the SV40 enhancer. pSV2-CAT was used as a positive control, and pSV1′-CAT, which lacks most SV40 enhancer (39), was used as a negative control. RNA from cells transfected with pSV2-CAT showed three bands of 100, 125, and 131 bp in length (Fig. 6, lane 1). The 125- and 131-bp bands corresponded two initiation sites reported earlier (8). The origin of the 100-bp band is not clear, but it may represent an early termination product. RNA from cells transfected with pSV1.6-CAT showed the same bands at a lower intensity (Fig. 6, lane 2). The insertion of the 409-bp Sd into pSV1.6-CAT resulted in

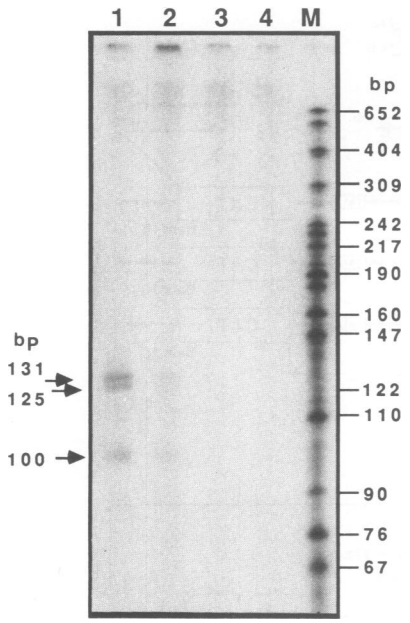


FIG. 6. Primer extension analysis of CAT transcripts in huH-1/cl-2. pSV2-CAT (lane 1), pSV1.6-CAT (lane 2), pSV1.6+409(N)-CAT (lane 3), and pSV1'-CAT (lane 4) were transfected into huH-1/cl-2 cells, and total RNAs were isolated 36 h later. Primer extension analysis was conducted as described in Materials and Methods. Lane M, pBR322 DNA digested with *Hpa*II, used as markers.

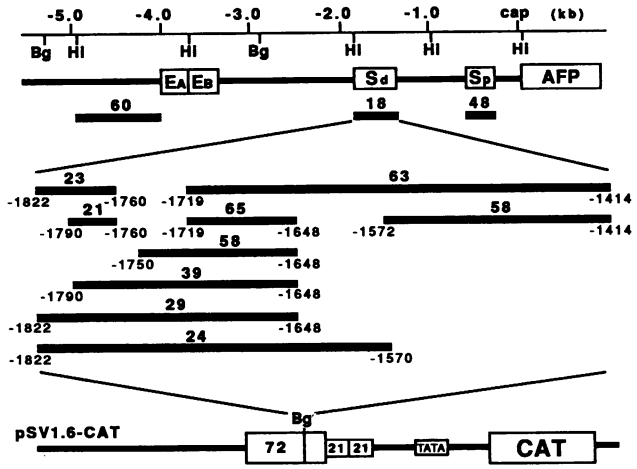


FIG. 7. Delimitation of the silencer region. Various fragments of the distal negative regulatory region, Sd, were inserted into the *Bgl*II site of pSV1.6-CAT. The precise endpoints of deletion are indicated in base pairs from the AFP cap site. These constructs were transfected into huH-1/cl-2 cells. CAT activities expressed are shown as percentages of the pSV2-CAT activity above the corresponding fragments. Suppression of CAT expression by the proximal negative region, Sp, was similarly analyzed. Hi, *Hin*dIII; Bg, *Bgl*II.

disappearance of these bands (Fig. 6, lane 3). No new bands were detected. Similarly, no bands were visible with RNA from cells transfected with pSV1'-CAT (Fig. 6, lane 4). These results show that Sd suppresses transcriptional initiation without altering the start site.

**Identification of a silencer element.** To delimit the Sd region that exerts transcriptional suppression, we inserted various lengths of Sd subfragments in the SV40 enhancer (*Bgl*II site) of pSV1.6-CAT (Fig. 7). Five fragments covering the region upstream of −1760 suppressed CAT activity. The shortest active fragment was 31 bp long, from −1790 to −1760. Four other fragments covering the region downstream of −1750 were inactive (Fig. 7). The 229-bp Sp region from −402 to −174 was also tested in a similar manner. It showed transcription-suppressive activity, although to a lesser degree than did Sd (Fig. 7).

We found that the 31-bp fragment which exhibited suppressive activity contains a 17-bp stretch, 5'-CTTCATAAC TAATACTT-3', which is repeated four times within a 90-bp region from −1810 to −1720 (Fig. 8A, underlined). A similar sequence is also found in the Sp region (Fig. 8B, underlined). In all cases, the first six nucleotides, CTTCAT, and the last two, TT, are completely conserved (Fig. 8C). DNase I footprinting analysis showed that these sequences were protected by nuclear extracts prepared from huH-1/cl-2 (data not shown).

To further characterize the function of these sequences, we inserted the 31-bp oligonucleotide within or upstream of the SV40 enhancer or downstream of the CAT gene in pSV1.6-CAT. The fragment inserted within the SV40 enhancer strongly suppressed CAT activity in huH-1/cl-2 (Fig. 9A, lane 8), but only a weak suppression was observed in HuH-7 (Fig. 9A, lane 2). The insertion of the fragment upstream of the SV40 enhancer (Fig. 9A, lanes 4 and 10) or downstream of the CAT gene (Fig. 9A, lanes 5 and 11) had no effect on CAT activity in either cell line. Substitution of three nucleotides within the 17-bp repeated sequence (Fig. 9B) resulted in loss of suppressive activity (Fig. 9A, lane 9). This finding confirms the association of the suppressive activity with this sequence.

To determine the relationship between transcriptional suppression and the number of copies of the Sd element, we inserted one, three, or eight copies of the 31-bp sequence into pAF5.1[Δ2.7]-CAT. Analysis of CAT expression from these constructs showed that CAT activity decreased with increasing number of copies of this sequence in huH-1/cl-2 but not in HuH-7 (Fig. 9C).

## DISCUSSION

Two human hepatoma cell lines, HuH-7 and huH-1/cl-2, are characterized by high and low levels of AFP gene expression, respectively. In this study, we show that the low AFP production in huH-1/cl-2 is not due to inactivation of the AFP enhancer or promoter or to drastic reduction of positive transcription factors such as NF-1/CTF and HNF1/LF-B1/AFP1. Our results showed that the reduced AFP expression in huH-1/cl-2 is due to about 4-fold reduction of the intrinsic AFP promoter activity and about 20-fold reduction of the stimulatory activity of the AFP enhancer. The reduced stimulatory activity of the AFP enhancer is due to activation of at least two silencer regions. Neither region is active in HuH-7. Further characterization of the strong, distal silencer, Sd, showed that (i) it blocks homologous and heterologous (SV40) enhancer activity in a position-dependent and orientation-independent manner, (ii) it has no direct suppressive effect on the AFP promoter, and (iii) it suppresses transcriptional initiation without altering the start site. These results indicate that Sd inhibits transcriptional

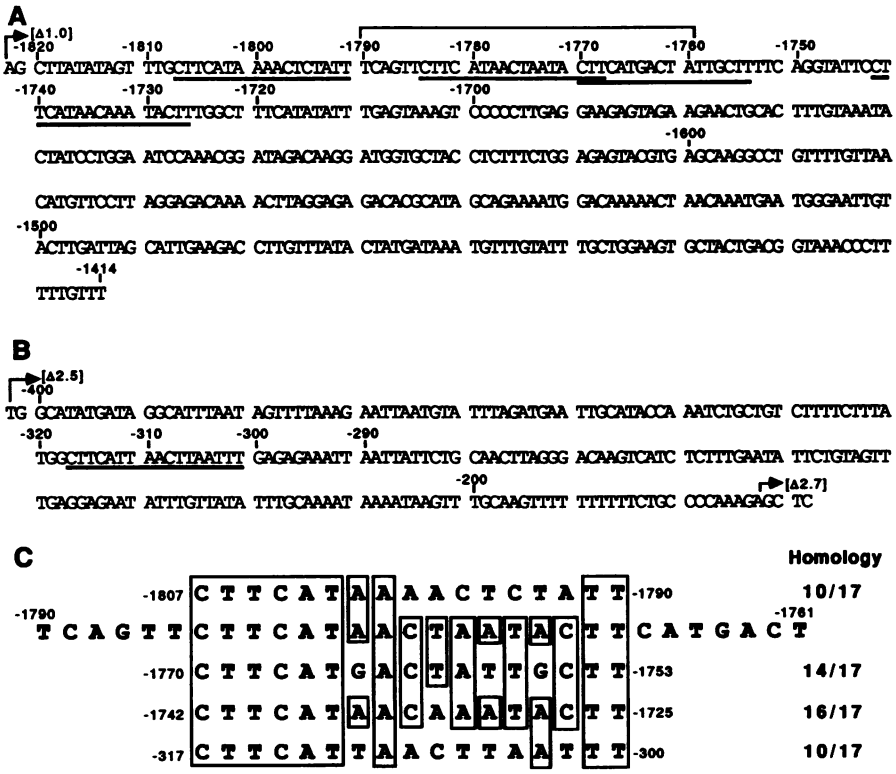


FIG. 8. Nucleotide sequence of the distal and proximal silencer regions. (A) Nucleotide sequence of Sd (from −1822 to −1414 bp). The 17-bp repeated elements are underlined. The 31-bp region used in CAT assays is shown by a bracket above the sequence. (B) Nucleotide sequence of Sp (from −402 to −169 bp) (31). The 17-bp repeated element is underlined. (C) Comparison of the 17-bp repeated elements in Sd and Sp regions. Identical nucleotides are boxed. The numbers of the matched nucleotides are shown on the right.

initiation by interfering with enhancer activity. We have identified a suppressor element, 5′-CTTCATAACTAATA CTT-3′, which is repeated four times in the Sd region and once in the Sp region. In all cases, the first six and the last two nucleotides (underlined) are conserved, suggesting the importance of these sequences in negative regulatory activity. Although this element is not strictly cell specific, it is highly specific to the AFP gene as a result of its dependency on the location relative to the enhancer and the promoter. This element appears to be distinct from other sequences proposed to down-regulate genes such as chicken lysozyme (2, 35), chicken vimentin (12), human ε-globin (6), mouse renin (28), mouse albumin (16), chicken cardiac myosin light-chain 2 (30), and rat glutathione transferase P (18) genes.

Muglia and Rothman-Denes (22) have shown that a 550-bp region located between the promoter and the enhancer (at −3.5 kb) of the rat AFP gene down-regulates transcriptional initiation from homologous and heterologous promoters. In contrast to the human AFP-negative element reported here, its function is limited to hepatoma cells. On the other hand, Vacher and Tilghman (38) have reported that a region between −838 and −250 bp of the mouse AFP gene suppresses AFP and albumin enhancer in a position-dependent manner in adult liver. Since this negative region is inactive in fetal liver, it is proposed that it may play an important role in developmental repression of the AFP gene. The complete nucleotide sequence of the mouse negative region is not available at present. Consequently, we could not firmly establish whether the same elements are involved in AFP gene repression in mice and humans. However, we note that a sequence similar to the Sp element is present at a corresponding site upstream of the mouse AFP gene. The mouse and human negative elements are also similar with respect to position dependency. It is therefore possible that the human silencer is also involved in developmental suppression of the AFP gene.

The presence of the silencer for the AFP gene has interesting implications with respect to differential regulation of the AFP and albumin genes. Camper and Tilghman (5) have reported that in transgenic mice, the AFP enhancer can stimulate the linked albumin promoter in adult as well as in fetal liver, raising the possibility that in adult liver, the AFP enhancer remains functional and stimulates the albumin gene. The silencers reported here and by Vacher and Tilghman (38) may function as a molecular switch to change the direction of enhancer action from the AFP to albumin promoter.

It is likely that the negative regulatory elements, like positive counterparts, function in conjunction with protein factors that interact with them (38). The binding of such proteins to the silencer element may interfere with the interaction of transcription factors with the enhancer or promoter or the formation of a DNA loop between these regulatory regions (29). Our preliminary experiments showed that Sd interacts strongly with huH-1/cl-2 nuclear extracts but weakly with HuH-7 nuclear extracts. We are conducting further analysis of the huH-1/cl-2 nuclear proteins interacting with Sd.

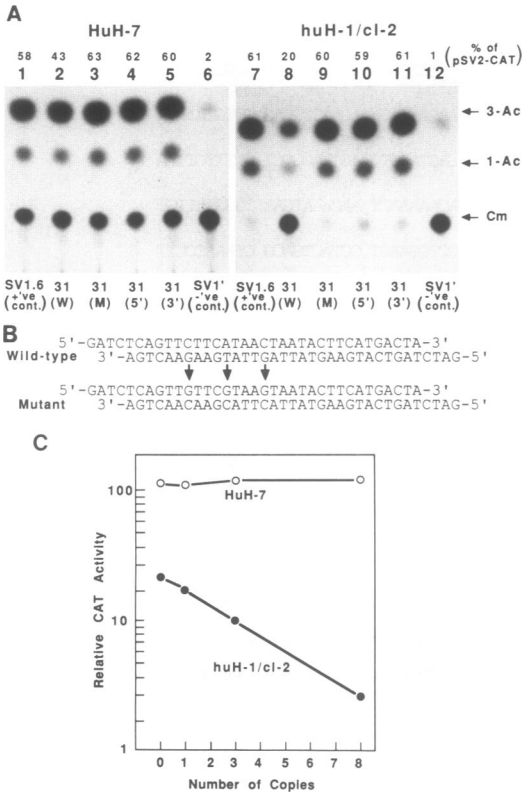


FIG. 9. Characterization of silencer activity associated with the 31-bp Sd fragment. (A) The 31-bp Sd fragment (from −1790 to −1760 bp) was inserted to the *Bgl*II, *Acc*I, or *Bam*HI site of pSV1.6-CAT (see Fig. 5A). A mutant fragment with three-nucleotide substitutions was inserted to the *Bgl*II site. These constructs were transfected into huH-1/cl-2 or HuH-7 cells, and 2 days later CAT activities were analyzed as described in Materials and Methods. The amounts of extract and the incubation times were 25 μg and 20 min for HuH-7 (lanes 1 to 6) and 100 μg and 180 min for huH-1/cl-2 (lanes 7 to 12). Cm, 1-Ac, and 3-Ac are as defined for Fig. 2B. Lanes: 1 and 7, pSV1.6-CAT (positive control); 2 and 8, wild-type 31-bp fragment inserted in *Bgl*II; 3 and 9, mutant fragment inserted in *Bgl*II; 4 and 10, wild-type fragment inserted in *Acc*I; 5 and 11, wild-type fragment inserted in *Bam*HI; 6 and 12, pSV1′-CAT (negative control). (B) Nucleotide sequences of the wild-type and mutant fragments. (C) Relationship between the number of copies of the 31-bp Sd fragment and transcription-suppressive activity. The 31-bp fragment was multimerized by self-ligation and inserted in the *Bgl*II site of pAF5.1[Δ2.7]-CAT, which was then transfected into HuH-7 or huH-1/cl-2 cells. CAT activities expressed are shown relative to that of pAF0.2-CAT (see Fig. 4).

## ACKNOWLEDGMENTS

We thank Tomonori Morinaga and Hisataka Yasuda for valuable discussion and technical advice and Howard Cheng and Wendy Matsumoto for technical help.

This work was supported by the National Cancer Institute of Canada, the Medical Research Council of Canada, and Japan Immunoresearch Laboratories, Co., Ltd. Taiki Tamaoki is a recipient of the Terry Fox Cancer Research Scientist Award of the National Cancer Institute of Canada.

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