The rat albumin gene promoter is appropriately regulated in transient but not in stable transfections

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ABSTRACT

The tissue-specific expression of the liver-specific rat albumin gene promoter was examined after transfer to various hepatic and non-hepatic cell lines. A 402 base pair sequence from the albumin gene 5' flank enabled a fused reporter chloramphenicol acetyltransferase gene to be expressed in rat hepatoma cell lines but not in fibroblast lines or dedifferentiated hepatoma cells. However, when this same construct was analyzed in permanently transfected cell populations, it was expressed equally well in differentiated and dedifferentiated hepatoma cells and in two of three fibroblast lines tested. The inappropriate expression of the albumin promoter was also seen using the HSV *tk* gene and the E. coli *gpt* gene as reporters, and when assayed by colony formation in HAT medium (*tk* gene) or by S1 protection of transcripts in cotransfected populations (*tk* and *gpt* genes). These results show that gene regulatory elements can behave differently in transient vs. stable transfections, and suggest that chromosomal integration can provide long range positive influences on gene expression.

INTRODUCTION

Recent advances in the identification of cis-regulatory elements of eukaryotic genes have generally been accomplished by the *in vitro* manipulation of cloned genes followed by the assay of their expression after transfection into cultured cells (1). Regulatory elements identified in such studies fall into two general categories, promoters and enhancers, although the distinction between the two is becoming increasingly hazy (1). The next step in an understanding of the regulation of gene expression is the identification of trans-acting factors that interact with these sequences. Footprinting and gel-shift assays have been used to reveal proteins (some tissue-specific and some ubiquitous) that bind to previously identified cis-regulatory elements of a number of genes (2,3,4,5,6); these assays provide a basis for the purification of trans-acting factors. The availability of *in vitro* transcription systems that mimic *in vivo* gene regulation has provided another means of identifying trans-acting factors (7,8,9). A third strategy for the identification of trans-acting regulatory factors is a genetic one. In this approach, a reporter gene

is joined to tissue-specific cis-acting regulatory sequences and introduced into cultured cells. The chimeric gene then acts as a stable and selectable marker for the detection of mutant or transfected trans-acting genes (10). The present work was undertaken with the aim of establishing such a system to study the regulation of the rat serum albumin gene.

The rat albumin gene is expressed exclusively in the liver of the adult animal. This specificity is reproduced in cell culture where cells derived from a minimal deviation hepatoma (H4IIEC3; hereafter, H4II cells) actively transcribe their albumin genes while cells of other histotypes do not (11). In addition, pleiotropically dedifferentiated variants of H4II cells have been isolated which cease to transcribe the albumin gene as well as other liver-specific genes (12,13). Hybrids formed between differentiated and dedifferentiated hepatoma cells or between differentiated hepatoma cells and non-hepatic cells do not express the albumin gene; this extinction of gene expression suggests the presence of a trans-acting negative factor(s) in non-differentiated cells (14). The introduction of a single, unique mouse fibroblast chromosome is sufficient for extinction of albumin expression in differentiated albumin-producing hepatoma cells, suggesting that a gene encoding a negative regulatory factor resides on this chromosome (15).

In order to provide a genetic assay for factors affecting albumin gene expression, we wished to construct stable rat hepatoma lines expressing a selectable gene under the control of the rat albumin promoter. It had previously been demonstrated that a 402 bp HincII fragment consisting of albumin sequences from -390 to +12 is sufficient to confer cell-type-specific expression on a heterologous gene when transiently transfected into cultured cells (16,17). A similar result was obtained after infections using an adenoviral vector containing albumin gene constructs (18,19). We therefore made plasmid constructions in which the 402 bp HincII fragment, or fragments extending farther 5', were fused to either the HSV tk gene, the E. coli gpt gene, or the chloramphenicol acetyltransferase (CAT) gene. These constructs were then transfected into albumin-producing and albumin-non-producing cell lines. Much to our surprise, although the albumin promoter was indeed inactive when assayed by transient expression in albumin-negative cell lines, it was fully active in most of these albumin-negative cell lines when introduced by stable transfection.

MATERIALS AND METHODS

Cell Culture

The high albumin producing line H4II (12) was a gift of M. Adesnik, the tk⁻ hepatoma line FT-1 (20) a gift of K. Fournier, mouse Ltk⁻a⁻ cells (21) a gift

of R. Axel, the rat liver fibroblast line Rat2 (22) a gift of C. Basilico, and the tk⁻ Chinese hamster ovary cell line 2F3 (23) a gift of L. Siminovitch. The dedifferentiated hepatoma line A1TG01 was previously described by Wolf et. al. (13).

All cell lines were maintained in Ham's F12 medium (24, 25) with 10% fetal calf serum (GIBCO). For selection of tk⁺ transformants the same medium was supplemented with dialyzed fetal calf serum and 500 nM amethopterin (HAT). G418 was used at 200-400 μ g/ml (GIBCO).

Plasmids and Phage

Charon 4A clone λ RSA30 (26) containing rat albumin sequences from about -8000 to +9000 relative to the transcription start site was a gift of T. Sargent. Plasmid pSV2gpt (27) was a gift of P. Berg, plasmids pM10TK (28) and pSVTK were gifts of E.D. Lewis and J. Manley, plasmid pNEOBPV (29) was a gift of M. Lusky, plasmids p Δ 232gpt and pESgpt (30) were gifts of D. Zaller and L. Eckhardt, and plasmids pSV2CAT and pSVOCAT (31) were gifts of C.M. Gorman.

Plasmid and Phage Constructions

Starting with albumin sequences contained in λ RSA30 and sequences from pSV2gpt, pM10TK, and pSV2CAT, plasmid constructions were made in which varying amounts of albumin 5'-flanking sequences were fused to the protein coding regions of the E. coli gpt, HSV tk, and CAT genes. In all cases a HincII site in the albumin flank at position +12 relative to the albumin transcription start site and -18 relative to the albumin translation start site was fused to a site upstream of translation initiation but downstream of transcription initiation in the reporter gene. Standard recombinant DNA methodologies were used throughout (32). The relevant features of the plasmids used are shown in Fig. 1. 5'-Deletions of pRSA1TK were made by digestion with SalI which cuts in the vector sequences 5' of the albumin flank, followed by BAL-31 exonuclease. The deleted genes were recloned so as to generate a series of mutants which were all joined at their 5'-end to the same the BamHI site in the vector. Gene fragments to be used as single-stranded probes in S1 protection analyses were cloned into the polycloning region of m13mp18 or m13mp19. The regions used as probes are included in Fig. 1.

Mammalian Cell Transfections

Plasmid DNA were prepared by standard procedures (32,33) and used to transfect cell cultures as a calcium phosphate coprecipitate (34). Each dish received a precipitate containing a total of 20 μ g of DNA. For experiments in which less than 20 μ g of plasmid DNA were used per dish, the total DNA amount was brought to 20 μ g with high molecular weight calf thymus DNA (Sigma). In transient transfections cells were refed nonselective medium about 20 hours

Figure 1. Plasmid constructs used. The open boxes represent the sequences of the particular reporter gene (HSV *tk*, Eco *gpt*, or CAT, as indicated in the construct name at the left). The thin line represents plasmid sequences. Albumin gene sequences are stippled, SV40 sequences are solid, and immunoglobulin gene sequences are cross-hatched. The arrow below each map shows the transcription start site. A stippled bar below a map represents a probe used in an S1 protection experiment, as described in the legends to Figures 2-5. Restriction sites: A, AccI; B, BamHI; G, BglII; E, EcoRI; H, HindIII; C, HincII; P1, PvuI; P2, PvuII; T, Tth111I; X, XbaI

later and harvested the next day (about 48 hours after the transfection was begun) for the preparation of CAT extracts.

S1 Protection Analysis

Total RNA was prepared by the method of Chirgwin et. al. (35). Uniformly labeled single-stranded probes were prepared as described by Burke (36). S1 mapping was performed essentially as described by Favalaro et. al. (37). Ten μ g and 50 μ g of total RNA were generally analyzed from each cell line or population. Ten μ g samples were brought to a total of 50 μ g RNA by the addition of 40 μ g of total RNA from nontransfected rat cells.

CAT Assays

CAT analysis was carried out essentially as described by Gorman et. al. (31).

RESULTS

Promoter Activity Measured by Colony Formation

The cell-type-specific activity of the rat albumin gene promoter was first tested by measuring its ability to drive the HSV tk gene. In these experiments the activity of the HSV tk gene was assayed by the ability of cells to form colonies in HAT medium, which requires TK enzyme activity. Two plasmids were constructed in which the albumin promoter was fused to the selectable HSV tk gene (Fig. 1). These constructs contained albumin sequences either from -390 to +12 (pRSA1TK) or from about -3800 to +12 (pRSA2TK) relative to the site of albumin transcription initiation. The albumin sequences of pRSA1TK have previously been shown to be sufficient for cell-type-specific activity when fused to the bacterial CAT gene and assayed in transient transfections (16,17). In both constructs the albumin sequences were fused to the HSV tk BglII site located at +52 relative to the HSV tk RNA cap site and -55 relative to the HSV tk translation start site. Transcription should thus be dependent on the albumin regulatory sequences and the peptide produced should be the wild type HSV TK protein. As a positive control we used pM10TK (Fig. 1; ref. 28) which contains the HSV tk gene under the control of its own promoter. As negative controls we constructed and used plasmids containing a promoterless HSV tk gene, pOTK (Fig. 1), or Δ 5-3, an inactive 5'-deletion mutant of pRSA1TK (see below).

Four tk⁻ cell lines were used as recipients in these experiments. As a cell with an active endogenous albumin gene, we used FT-1, a tk⁻ rat hepatoma

TABLE 1: ACTIVITY OF THE ALBUMIN PROMOTER IN STABLE COLONY ASSAYS

	FT-1	Rat2	Ltk ⁻ a ⁻	CHO2F3
Construct: pRSA1TK	0.86±0.17 (8)	8.17±0.93 (7)	-	-
pRSA2TK	0.74±0.14 (11)	5.79±0.69 (8)	1.83±0.28 (6)	0.12±0.06
pM10TK	0.39±0.09 (13)	6.44±0.65 (11)	3.88±1.20 (6)	1.66±0.19
pOTK	<0.01 (2)	<0.04 (1)	-	<0.03
pΔ5-3	<0.01 (2)	<0.04 (2)	0.18 (3)	<0.02
Relative Activity: pRSA1TK/pM10TK	2.2	1.3	-	-
pRSA2TK/pM10TK	1.9	0.9	0.5	0.07

The values shown are the average transformation frequencies, (colonies/cell/μg construct)×10⁴, ± the standard error, with the number of experiments shown in parentheses.

cell line (20). As judged by quantitative S1 nuclease analysis of steady-state RNA levels (data not shown), this tk⁻ line expresses its endogenous albumin gene at a relatively low rate, about 1% of the level found in the related (tk⁺) hepatoma line H4II. FT-1 cells do exhibit hepatic differentiation by several other criteria however (20). Three tk⁻ albumin-non-producing fibroblast lines were also transfected: Rat2 (22), mouse Ltk⁻a⁻ (21), and Chinese hamster ovary cell line 2F3 (23). The transfected cultures were plated in HAT medium to select for tk⁺ transfectants. The results of a number of transfection experiments are summarized in Table 1. It is clear from these experiments that by this test the albumin promoter is expressed in Rat2 and Ltk⁻a⁻ cells about as well as the HSV tk promoter, and that in FT-1 it is expressed only slightly (~2 times) more efficiently. A promoter is in fact necessary for expression, since the two promoterless constructs displayed less than one-tenth the activity of their counterparts containing the albumin promoter. Thus the albumin promoter exhibits considerable activity in these two albumin-negative lines. A different result was obtained with Chinese hamster 2F3 cells. The activity of the albumin promoter was only 7 percent of the activity of the HSV tk promoter in these cells.

The scoring of colonies may not be a sensitive indicator of promoter activity, since only a low level of tk mRNA may suffice for colony formation in HAT. Furthermore, despite the dependence on promoter sequences, there is no assurance from these experiments that the chimeric transcripts are being properly initiated in the albumin-negative lines. To address these issues, total RNA from pooled HAT-resistant Rat2 transfectants was analyzed by S1-protector

Figure 2. S1-mapping of *tk* RNA molecules produced in pooled populations of Rat 2 cells transfected with pRSATtk, pRSA2TK, and pM10TK. A 900 base pair XbaI-SphI fragment from pRSA2TK which spans the site of the gene fusion was subcloned into m13mp18 and used to make a uniformly labeled, single-stranded probe (mRSXTK, see Fig. 1) as described in Materials and Methods. RNA initiated at the proper site in the albumin promoter should protect 458 bases of this probe. RNA molecules initiated from the HSV *tk* promoter of pM10TK should protect 446 bases of the probe. Fifty μ g of total RNA from populations of *tk*⁺ Rat2 transformants were analyzed by S1 protection analysis. The 458 base fragment corresponding to pRSATtk and pRSA2TK transcripts (ALB) are indicated as is the 446 base fragment protected by transcripts from pM10TK (HSV).

tion. Protected fragments of 458 and 446 bases should be generated by properly initiated transcripts from the albumin and HSV *tk* promoters respectively. As can be seen in Figure 2, such fragments are in fact generated, indicating that the albumin promoter directs proper initiation of transcription in this albumin-negative fibroblast line. A comparison of the intensities of the bands in Fig. 2 reveals that the levels of properly initiated pRSATtk and pRSA2TK transcripts are similar to those of pM10TK transcripts in this cell line.

Thus, in contrast to previously reported results using transient transfections (16,17) or viral vector infections (18,19), the albumin promoter is active in at least some albumin-negative cell lines in stable transfections.

Promoter Activity in Cotransfection Experiments

In the colony assays described above we were unable to use as a recipient the high albumin-producing hepatoma line H4II, since that line is *tk*⁺. Although the albumin promoter was clearly inappropriately active in albumin-negative lines it was possible that this promoter activity would be much greater in a high level albumin producer like H4II. In the colony assays we were also unable to use dedifferentiated hepatoma cell variants as recipients, again because no *tk*⁻ derivatives were available. Since the endogenous albumin gene has been silenced by a recent event in such variants, it is possible that an exogenous albumin promoter would also be silent in such cells. We therefore turned to non-selective methods for the analysis of the albumin promoter

Figure 3. S1-mapping of *gpt* RNA molecules produced in pooled populations of cells cotransfected by pNEOBPV and either pSV2*gpt* or albumin-*gpt* gene fusions. The 770 base pair XbaI-KpnI fragment of pJA1*gpt* was subcloned into ml3mp19 and used to make a uniformly labeled single-stranded probe as described in Materials and Methods. RNA initiated at the proper site in the albumin promoter should protect a fragment 212 bases long. RNA molecules initiated from the SV40 promoter should protect a 200 base fragment. The probe (mJAHK) is shown in Fig. 1. Ten μ g (odd numbered lanes) and 50 μ g (even numbered lanes) of total RNA from populations of G418-resistant cotransfectants which had been treated with pJA1*gpt* (A1TGO1 and H4II panels, lanes 1 and 2), pJA4*gpt* (A1TGO1 and H4II panels, lanes 3 and 4; other panels, lanes 1 and 2), or pSV2*gpt* (A1TGO1 and H4II panels, lanes 5 and 6; other panels, lanes 3 and 4) were analyzed. Closed arrows: the 212 base fragment protected by transcripts properly initiated in the albumin promoter. Open arrows: the 200 base fragment protected by transcripts initiated in the SV40 promoter. Lane M contains end-labeled HaeIII-digested ϕ X174 DNA as molecular size markers.

in stable transfectants. In these experiments, various chimeric constructs (with albumin or control promoters) were co-transfected into wild-type cells along with a plasmid (pNEOBPV, ref. 29) carrying a *neo* gene conferring resistance to the drug G418. G418-resistant colonies were then selected, pooled and analyzed for the expression of the chimeric genes by S1 nuclease protection.

One set of gene fusions utilized the *E. coli gpt* gene as a reporter. As shown in Fig. 1, they contained albumin gene flanking sequences from either -450 to +12 (pJA1*gpt*) or from -8000 to +12 (pJA4*gpt*). The latter contains all

TABLE 2: RELATIVE ACTIVITY OF THE ALBUMIN PROMOTER IN STABLE COTRANSFECTIONS

CELL LINES	pRSA2TK/pM10TK	pRSA2TK/pSVTK	pJA1gpt/pSV2gpt	pJA4gpt/pSV2gpt
H4II	0.61 (1)	0.52 (1)	1.23 (2)	0.90 (3)
A1TG01	0.72 (1)	1.06 (1)	1.67 (2)	1.75 (3)
Rat2	0.51 (1)	-	-	1.46 (2)
Ltk ^{-a-}	-	-	-	3.18 (2)
CHO 2F3	-	0.045 (2)	-	0.02 (2)

Various cell lines were cotransfected with pNEOBPV and a gpt or tk construct. G418-resistant colonies were selected and pooled and total RNA was analyzed by S1 analysis as exemplified by the data in Figures 2 and 3. The activities of the various promoters were determined by densitometry. Ratios were calculated for different dishes transfected on the same day. Values are averages of different experiments, the number of which is shown in parentheses.

of the albumin 5'-flanking sequences previously cloned (26). As a positive control cells were independently cotransfected with pSV2gpt in which the gpt gene is under the control of SV40 early regulatory sequences. The S1-protection experiments were performed using a probe containing sequences from pJA1gpt that span the joint of the gene fusion (mJAHK, Fig. 1). Properly initiated transcripts from pJA1gpt and pJA4gpt should protect 212 bases of this probe whereas transcripts initiated from the SV40 promoter of pSV2gpt should protect a 200 base fragment. These constructs were cotransfected into the high albumin-producing hepatoma line H4II, the dedifferentiated albumin-negative hepatoma line A1TG01, Rat2 fibroblasts, mouse Ltk^{-a-} fibroblasts, and Chinese hamster 2F3 fibroblasts. As can be seen Figure 3, transcripts properly initiated from the albumin promoter exist in transfected populations of all tested cell lines (solid arrows). The level of such transcripts is similar to the level of transcripts generated by pSV2gpt (open arrows) in parallel transfections of all of these cell lines except Chinese hamster 2F3 (the activity of the SV40 promoter in H4II cells in the experiment shown in Fig. 3 was anomalously high: see Table 2.) The RNA levels in these experiments were quantified by densitometry and the results are shown in Table 2. Also shown in Table 2 are the results of similar experiments using the albumin-HSV tk fusions described above, with either the HSV tk or SV40 early promoter as a positive control. In all cell lines except 2F3 the albumin promoter is at least as active as either the HSV tk promoter or the SV40 early regulatory region. In 2F3 cells the albumin promoter is only about 3% as active as the SV40 promoter. This cell line was also the only one in which the albumin promoter was

not efficiently used when tested in the colony assay (see Table 1). It should also be noted that the relative activities of pJAlgpt (450 bp of albumin 5' flank) and pJA4gpt (~8000 bp of albumin 5' flank) were similar. The lack of regulation of the albumin promoter (or its regulation in 2F3 cells) could not be explained by differences in copy number of the different constructs: Southern analysis of transfected populations revealed no consistent differences in the relative copy numbers of albumin-gpt vs. pSV2gpt or albumin-tk vs. HSV tk in the albumin-negative lines compared to H4II (data not shown). To test the idea that the activity of the albumin promoter in albumin-negative lines could be accounted for by the presence of a few clones expressing the gene fusions at a high level, we also analyzed individual clones of albumin-negative A1TG01 cells cotransfected with pNEOBPV and either pM10TK, pRSA1TK, or pRSA2TK. All clones that contained tk DNA expressed it and the levels of expression were somewhat greater in clones transfected with pRSA1TK or pRSA2TK than in those transfected with pM10TK (4 to 6 clones of each type analyzed, data not shown). The inappropriate expression observed also could not be explained by a change in the albumin phenotype of transfected cells: S1 analysis showed that the endogenous albumin gene was still active in G418-resistant H4II co-transfectants and remained inactive in co-transfected populations of Rat2 and A1TG01 cells (data not shown). We therefore conclude that the albumin promoter was inappropriately active in three of four albumin-negative cell lines as assayed in stable transfectants by colony formation or by cotransfection.

Enhancer Requirement in Cotransfection Assays

We wanted to know if the inappropriate activity of the albumin promoter was specific to this promoter or was a general consequence of the use of the stable cotransfection assay. Toward this end we made use of two additional constructs, pΔ232gpt and pESgpt (Fig. 1). pΔ232gpt is a derivative of pSV2gpt in which SV40 sequences upstream of position 114 on the standard SV40 map have been deleted. The SV40 early transcription initiation sites and the 21 base pair repeats are retained but most of the 72 bp repeats are no longer present. This deletion is known to eliminate SV40 early expression (38). pESgpt contains the immunoglobulin heavy chain enhancer inserted into pΔ232gpt just upstream of the deletion (30). These constructs were used to stably cotransfect albumin-negative A1TG01 cells. As a positive control these cells were cotransfected with pSV2gpt. Total RNA from pooled transfectants was analyzed by S1-protection as shown in Fig. 4. Much to our surprise, pΔ232gpt, pESgpt, and pSV2gpt were equally active in A1TG01 cells (Fig. 4, lanes 1-3 and 6-7). Thus an enhancer is not required for efficient use of the SV40 early promoter in

Figure 4. Enhancer requirement in cotransfection assays. The 320 bp SphI-BglII fragment of pSV2gpt which includes the early transcription initiation sites of SV40 was subcloned into m13mpl9 and used to make a uniformly labeled, single-stranded probe as described in Materials and Methods. RNA transcripts properly initiated from pSV2gpt should protect fragments ranging in size from 179 bases to 188 bases. The probe (mSVgpt) is shown in Fig. 1. Fifty μ g of total RNA from populations of G418-resistant cotransfectants of A1TGO1 cells (lanes 1-3 and 6-7) or CHO 2F3 cells (lanes 4-5) were analyzed by S1 protection. In addition to pNEOBPV, cells were transfected with pSV2CAT (lanes 1, 4, and 6), pESgpt which contains the immunoglobulin heavy chain enhancer in place of the SV40 enhancer (lanes 2, 5, and 7), or p Δ 232 which lacks an enhancer (lane 3).

this cell line when assayed in a stable cotransfection assay.

We next tested for an SV40 enhancer requirement in Chinese hamster 2F3 cells. Of the four albumin-negative cell lines tested above, only in 2F3 cells was the albumin gene promoter appropriately inactive. In this case we compared the activity of pSV2gpt and pESgpt; in the latter plasmid the immunoglobulin enhancer has been substituted for the SV40 enhancer. As can be seen in Fig. 4 (lanes 4 and 5), pSV2gpt is much more active than pESgpt in 2F3 cells. Thus, among the cell lines studied, only 2F3 cells responded as expected to stably transfected albumin and pESgpt regulatory elements.

Albumin Promoter Sequence Requirements for Expression in A1TGO1 Cells

The ability of the SV40 promoter to function in A1TGO1 cells in the absence of an enhancer, led us to consider whether a simple promoter sequence (devoid of any upstream regulatory elements) was all that was required for the expression of transfected genes in these cells. To define the 5' sequence requirements for the activity of the transfected albumin promoter, we made several 5' deletion mutants of pRSa1TK. These mutant DNA preparations were cotransfected with both pNEOBPV and pM10TK into A1TGO1 cells. G418-resistant colonies were pooled and total RNA from each population analyzed by S1-protection as in Fig-

TABLE 3: EFFECT OF 5' DELETIONS ON THE ACTIVITY OF THE ALBUMIN PROMOTER

CONSTRUCT (bp of 5' flank)	AVERAGE % pM10TK ACTIVITY
pRSA2TK (-2000)	117
pRSA1TK (-390)	97
pΔ5-217 (-350)	88 ±9
pΔ5-1 (-135)	80 ±1.5
pΔ5-2 (-105)	25 ±1
pΔ5-3 (-63)	<10

Total RNA from pooled G418-resistant ALTGO1 populations was analyzed by S1 protection using probe mRSXTK (see Fig.1). In addition to pNEOBPV, cells were transfected with 2 µg/dish of pM10TK as an internal control and 5 µg/dish of the albumin deletion plasmids indicated above. Autoradiographic bands were quantified by densitometry and the activity of the albumin promoter in each lane determined relative to the activity of the HSVTK promoter in the same lane. For the deletion mutants, two independent pooled populations were assayed; the average and standard error of these measurements are given.

ure 2. Transcripts initiated from the HSV tk promoter of pM10TK could be distinguished by size from those initiated from the albumin promoter of the deletion mutants; the HSV tk transcripts served as an internal control for promoter activity. The mutants contained albumin sequences to approximately -350, -135, -105, and -63 relative to the albumin cap site (mutants pΔ5-217, pΔ5-1, pΔ5-2, pΔ5-3, respectively). It is apparent from Table 3 that activity of the albumin promoter in these stable transformants is dependent on sequences downstream of position -135. pΔ5-2, which contains sequences only to -105, has reduced activity. The region deleted in this mutant includes a sequence conserved among rat, mouse, human, and chicken albumin genes (16). In mutant pΔ5-3, 63 base pairs remain 5' of the transcriptional start site, and a CAAT box (16) has been deleted; this mutant yields no detectable promoter activity. These results indicate that the inappropriate activity of the albumin promoter in at least ALTGO1 remains dependent on upstream regulatory sequences.

Transient CAT Assays

The results of these stable transcription assays apparently contradict those of Ott et. al. (17) and Heard et. al. (16) who found appropriate regulation of the albumin promoter in transient CAT transfection experiments. To explore the basis of this discrepancy, we carried out similar transient transfection experiments. We fused the albumin promoter (-390 to +12) to the bacterial CAT gene and tested its activity, relative to pSV2CAT, in both stable

TABLE 4. CAT ACTIVITIES IN TRANSIENT AND STABLE TRANSFECTIONS

	H4II	AltG01	Rat2	CHO2F3
TRANSIENT:				
pRSA1CAT	6.0 $\pm$ 2.0 (12)	3.3 $\pm$ 0.6 (19)	2.4 $\pm$ 0.8 (2)	1.7 $\pm$ 0.5 (2)
pSV2CAT	9.2 $\pm$ 1.4 (13)	107 $\pm$ 14 (20)	80 $\pm$ 17 (2)	126 $\pm$ 2 (2)
pSVOCAT	0.2 $\pm$ 0.03 (3)	1.6 $\pm$ 0.0 (2)	2.1 $\pm$ 0.7 (2)	2.8 $\pm$ 0.6 (2)
<u>pRSA1CAT</u>				
pSV2CAT	0.65	0.03	0.03	0.014
STABLE:				
pRSA1CAT	13.6 $\pm$ 0.0 (2)	112 $\pm$ 24 (6)	11.4 $\pm$ 0.2 (2)	1.1 $\pm$ 0.7 (3)
pSV2CAT	9.6 $\pm$ 0.8 (2)	273 $\pm$ 27 (5)	6.8 (1)	226 $\pm$ 100 (3)
pSVOCAT	<2.4 (2)	<3.0 (2)	0.8 $\pm$ 0.2 (2)	<0.2 (2)
<u>pRSA1CAT</u>				
pSV2CAT	1.42	0.41	1.68	0.005

Activities presented are the percent chloramphenicol acetylated per 200 μ g soluble protein per 2 h incubation in the transient transfections, and per 0.5 h incubation in the stable transfections. Average values are given $\pm$ the standard error, with the number of independent transfections in parentheses.

and transient transfection experiments. In the transient assays, cells were transfected with 20 μ g of the CAT construct and extracts prepared 48 hours later. In the stable assays, cells were cotransfected with a CAT construct and pNEOBPV, and G418-resistant colonies were selected and pooled. CAT enzymatic activity was then measured in extracts made from the pooled populations. The results of such transfections in H4II, AltG01, Rat2, and 2F3 cells are summarized in Table 4. In the transient assay, the albumin promoter was essentially inactive (i.e. similar in activity to the pSVOCAT promoterless control) in all cell lines except H4II. In H4II the albumin promoter was about as active as the SV40 promoter. The result was markedly different in the stable assay. In this case the albumin promoter exhibited substantial activity in all lines except 2F3. Thus in the stable CAT assay, as in the other stable assays, the albumin promoter was inappropriately active in all albumin-negative lines except 2F3. We therefore conclude that the behavior of the albumin gene promoter is dependent on both the assay used (stable or transient) and the cell line into which it is introduced.

DISCUSSION

We initially set out to generate cell lines containing chimeric genes under the control of the albumin promoter. It was anticipated that in such lines

regulation of the exogenous albumin promoter would parallel that of the endogenous albumin promoter. Specifically, we expected the chimeric genes to be expressed only in albumin-producing cell lines. This expectation was bolstered by the fact that similar chimeric constructs had been reported to be expressed exclusively in albumin-producing cell lines in both transient transfection and infection experiments (18,19,16,17). We were therefore surprised to find that in stable transfections the albumin promoter was as active in three albumin-negative cell lines as in albumin-positive lines. Inappropriate expression was obtained whether activity of the albumin promoter was directly selected or whether it was measured after cotransfection with an independently selectable marker. S1-protection analysis revealed that in all cases transcription was properly initiated. DNA copy number measurements in the transfected lines and populations showed that the activity in negative cells could not be accounted for by the inefficient expression of many gene copies. Inappropriate activity was observed whether the reporter gene was the HSV tk, E. coli gpt, or bacterial CAT gene and whether activity was normalized to the SV40 early promoter and enhancer or to the HSV tk promoter. In contrast to these results, proper regulation was observed when a transient transfection assay was used. The same albumin-CAT gene fusion that was properly regulated in the transient assay was inappropriately active when stably cotransfected into albumin-negative lines; thus proper regulation is apparently dependent on the type of transfection (stable or transient) and not on the construct used.

One model to account for these results would be to postulate that small amounts of a positively-acting transcription factor exist in many albumin-negative cell types. This small amount would be sufficient for full activation of the few copies of the gene introduced by stable transfection. However, it would be insufficient to fully activate the perhaps thousands of gene copies introduced in the transient assay. Albumin-positive cells would contain much greater amounts of this factor, enough to fully activate even the large number of transiently transfected genes. However, this model does not explain why the two endogenous albumin genes do not respond to this putative activator in albumin-negative cell lines.

Another way to explain our results is based on the fact that in stable, but not transient, transfections, the exogenous DNA is integrated into the cellular genome (39). It is therefore possible that regulation of the albumin promoter is affected by neighboring host or carrier DNA sequences. Unlike some tissue-specific elements, the specificity of the albumin promoter can be overridden by linkage to the SV40 enhancer (16). Thus the albumin promoter

may be more sensitive to the influence of neighboring sequences than the promoters of other tissue-specific genes which have been studied. In the case of the cotransfections, the albumin promoter could have been influenced as well by the selectable marker gene (pNEOBPV) employed. We think this explanation is unlikely because: 1) in most transfectants the albumin promoter should be separated from the cotransfecting neo gene by the excess of carrier (calf thymus) DNA included; and 2) alb-tk constructs were efficient in transforming tk⁻ cells to HAT-resistance in the absence of any other plasmid (Table 1); the inclusion of pNEOBPV did not significantly increase the frequency of transformation (data not shown). The pNEOBPV could be indirectly affecting the results: by selecting for the activity of the neo gene, we may be selecting for integration of all transfecting DNA into active chromatin regions. Indeed, nonselected, cotransfected DNA, even when prokaryotic in origin, has been shown to be in a potentially active state as assayed by its accessibility to a variety of nucleases and its association with active supranucleosomal particles (40,41). However, this explanation cannot account for the inappropriate activity of the albumin promoter in stable colony assays since no cotransfecting DNA was used. A more extreme version of this idea is that in stable transfections the integrated DNA is usually assembled into active chromatin irrespective of selective pressure. There is some evidence that transfected genes are in a different state from their endogenous counterparts. For instance, the mutation frequency for transfected genes is generally much higher than that for endogenous genes (42, 39). Furthermore, whereas deletions account for only a small fraction of spontaneous mutations of endogenous genes (43), deletions are the predominant form of mutation for transfected genes (39).

The activity of the albumin promoter in albumin-negative cell lines still required more than 63 bp of albumin flank. For maximal activity 135 bp were required. Thus factors capable of productively interacting with the albumin flank must be present in three of the four albumin-negative lines tested. In fact, in vitro footprinting and gel-shift studies have shown albumin gene-specific binding factors in extracts from albumin-non-producing cells (2,44,45). These factors may not be the same as those responsible for transcriptional activity in albumin-producing cells. The endogenous albumin gene as well as transiently transfected albumin promoters are obviously not responding to these non-specific factors. Perhaps these genes are, for some reason, less accessible.

The albumin promoter was not the only regulatory element to be inappropri-

ately regulated in our stable transfection experiments. In A1TG01 cells, the SV40 early promoter was expressed equally with or without an enhancer. This was not the case in 2F3 cells - the one albumin-negative line in which the albumin promoter was appropriately inactive. Thus, either exogenous DNA is not generally packaged into potentially active chromatin in 2F3 cells or these cells lack factors required for the activation of both the albumin and SV40 early promoters.

The fact remains that a number of tissue-specific genes are appropriately regulated in stable transfections (46,47,48,49), although inappropriate regulation has been observed for others (50,51). Why is the albumin promoter not appropriately regulated in these albumin-negative lines? One possibility is suggested by the results of Deschattrette et al. (52). In contrast to our results, they found appropriate regulation of the mouse albumin promoter in stable transfections of differentiated and dedifferentiated rat hepatoma cells. In their experiments, the entire mouse albumin gene was used, including all introns and 4.3 kb of 3' flanking sequence in addition to 2.2 kb of 5' flank. These additional sequences may be required for proper regulation in the stable assay. There is also evidence that as much as 10 kb of 5'-flank is required for proper regulation of the mouse albumin promoter. Pinkert et. al. (53) reported the presence of an enhancer 10 kb upstream of the transcription start site of the mouse albumin gene. Chimeric genes lacking this sequence were only one-tenth as active when assayed in transgenic mice, and in some mice were inappropriately active in the kidney. Thus it is possible that the rat albumin gene also contains regulatory sequences more than 8 kb upstream of transcription initiation. The role of such additional elements (whether in flanks or in introns) may be to prevent neighboring sequences from organizing the assembly of integrated DNA into active chromatin. In transiently transfected cells these additional sequences presumably would not be necessary, since there are no neighboring sequences to organize the transferred DNA. Thus, stable transfection may allow identification of regulatory signals responsible for proper chromatin assembly; signals that may not be required for proper regulation in transient transfections.

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