Tissue-Specific in Vitro Transcription from the Mouse Albumin Promoter

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Tissue-Specific In Vitro Transcription from the Mouse Albumin Promoter

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Summary

Transcriptionally active nuclear extracts have been prepared from rat liver, brain, and spleen. The adenovirus-2 major late promoter directs efficient transcription by RNA polymerase II in all of these extracts, whereas the promoter of the mouse albumin gene is significantly used only in the liver extract. Albumin sequences located between −170 and −55 are required for this liver-specific in vitro transcription, since deletion of this region results in almost a 100-fold reduction in transcription. In addition, insertion of these sequences in either orientation upstream of the parotid-specific *Amy-1* promoter, which is poorly transcribed in the liver extract, increases the activity of this promoter to a level comparable to that observed for the albumin promoter.

Introduction

In vitro transcription systems are important for deciphering the precise molecular mechanisms underlying selective transcription initiation. Thus far most RNA polymerase II in vitro transcription extracts have been prepared from mammalian (reviewed by Heintz and Roeder, 1982; Manley, 1983) or Drosophila (Parker and Topol, 1984a) tissue culture cells. These extracts have been shown to be capable of correctly initiating transcription at promoters of numerous cellular and viral genes and have been used successfully for studying general transcription initiation factors, such as the TATA binding factor (Wu, 1985), as well as proteins involved in the assembly of preinitiation complexes (Fire et al., 1984). Several reports have been published on in vitro transcription involving promoter-specific initiation factors. These include Sp1, a DNA-binding protein recognizing the sequence GGGCGG, which is present in the simian virus 40 (SV40) early promoter as well as in promoters of certain cellular genes (Gidoni et al., 1985; Jones et al., 1985); heat-shock transcription factor, a factor binding to a conserved sequence upstream of Drosophila heat-shock genes (Parker and Topol, 1984b); and a protein augmenting adenovirus-2 major late transcription by binding to a region upstream of the TATA box (Sawadogo and Roeder, 1985b; Carthew et al., 1985). (For reviews of these and other transcription factors see Dynan and Tjian [1985] and Hansen and Sharp [1984].)

The cell types generally used as a source for extracts are poorly differentiated, and only a few reports have suggested that tissue specificity may be operating in vitro (Tsuda and Suzuki, 1981; Bazett-Jones et al., 1985; Schöler and Gruss, 1985). To our knowledge, there have been no reports on the preparation from mammalian organs of transcriptionally active extracts that mimic tissue-specific transcription.

We have chosen the promoter of the mouse albumin gene as a model for our studies of tissue-specific in vitro transcription. The primary structure of the albumin promoter region has been determined in several species of mammals, including humans (Minghetti et al., 1986), rats (Urano et al., 1986), and mice (Carneiro, 1985), and the developmental and tissue-specific expression of this gene has been studied in detail (Tilghman and Belayew, 1982; Krumlauf et al., 1985; Godbout et al., 1986). The mouse albumin gene is expressed primarily in the liver, where it is coordinately activated with the closely linked α-fetoprotein gene during the fetal development of this tissue. After birth α-fetoprotein expression rapidly decreases by approximately four orders of magnitude from its peak level attained 2 days before birth. In contrast, albumin transcription continues to increase after birth until, at 3 weeks, the albumin mRNA concentration reaches adult levels (Tilghman and Belayew, 1982). Whereas the mouse albumin gene is among the most efficiently expressed liver-specific genes, fewer than 100 molecules per cell of albumin mRNA are produced in brain, kidney, and pancreas, and the gene is completely silent in the salivary gland and spleen (M. C. and U. S., unpublished observations). Transfection experiments with fusion genes containing the albumin 5′-flanking region have shown that these sequences confer tissue specificity to the expression of such hybrid genes (Ott et al., 1984). Comparison of albumin 5′-flanking sequences (from +1 to −116) from mouse (Carneiro, 1985) and rat (Sargent et al., 1981) shows more than 90% sequence conservation between these two rodent species. It is therefore not surprising that, in transfection studies, these promoter sequences are faithfully recognized across species (Deschatrette et al., 1985).

In this paper we report the preparation of transcriptionally active soluble protein extracts from purified nuclei of several rat tissues, and demonstrate tissue-specific transcription from the mouse albumin promoter in vitro. Moreover, we define a sequence that is required for efficient in vitro transcription from the albumin promoter in a liver extract.

Results

Construction of Templates for In Vitro Transcription

In vitro, RNA polymerase II does not terminate transcription properly, even in systems that are able to initiate and elongate the transcripts accurately (reviewed by Manley, 1983). For this reason, in most in vitro transcription assays a truncated template is used in order to generate a discrete run-off transcript. Alternatively, fairly time-con-

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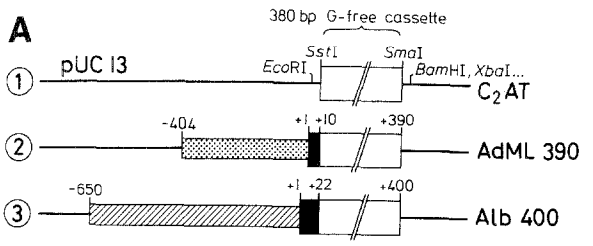

Figure 1. Transcripts Synthesized In Vitro from the AdML and Albumin Promoters

(A) Diagram of the G-free cassette vector $p(C_2AT)_{19}$ and the two promoter-containing constructs. The 5′-flanking region of each gene is shown either stippled (AdML) or with diagonal lines (albumin) and the sequences downstream of the cap site are represented in black.

(B) In vitro transcription products. The three templates $p(C_2AT)_{19}$ (lane 1), AdML390 (lane 2), and Alb400 (lane 3) were assayed with 45% extract by volume (2.9 mg/ml protein in the reaction) as described in Experimental Procedures.

suming methods, such as nuclease S1 mapping and cDNA primer extension, must be employed to detect the accurately initiated transcripts from supercoiled templates. The vector $p(C_2AT)_{19}$ (Sawadogo and Roeder, 1985a) was designed to surmount this difficulty in an elegant way. It contains a synthetic run of 380 A, C, and T residues on the messenger strand. When a promoter fragment that has no G residues downstream of the cap site is cloned directly upstream of this "G-free cassette," an in vitro transcription template results that, in the absence of GTP, generates a specific transcript whose 5′ and 3′ boundaries are determined by the cap site and the first G residue flanking the cassette, respectively. Apart from their use in rapid transcription assays in which radiolabeled transcripts are simply precipitated with trichloroacetic acid and the amount of label incorporated is directly determined (Sawadogo and Roeder, 1985a), such constructs have several additional advantages. First, the efficiency of circular and linear templates in promoting accurate transcription initiation can be directly compared. Second, nonspecific transcripts, such as those resulting from end-to-end transcription of restriction fragments or from initiation at pseudopromoters, are not successfully elongated. Third, symmetrical transcription, which may possibly interfere with transcription from the promoter of interest, is largely abolished through use of the G-free cassette. All of these properties help to increase the ratio of specific to nonspecific transcripts, which is particularly important if specific transcription efficiencies have to be compared over a wide range.

Using the vector $p(C_2AT)_{19}$, we constructed a plasmid containing the promoter of the mouse albumin gene (base pairs −650 to +22) upstream of the G-free cassette. This template, referred to as Alb400, should generate a transcript approximately 400 nucleotides long. As a positive control for the transcriptional competency of our extracts, we have used the same G-free cassette under the direction of the adenovirus-2 major late (AdML) promoter (Sawadogo and Roeder, 1985a; this plasmid is referred to in their publications as $pML[C_2AT]_{19}$), which is a very strong promoter in most in vitro systems. This template, termed AdML390 herein, contains base pairs −404 to +10 of the viral promoter and generates a transcript 390 nucleotides long. In some experiments, the vector $p(C_2AT)_{19}$ was used as a negative control. Figure 1A schematically represents these three constructs.

Preparation of In Vitro Transcription Extracts from Solid Tissues

To isolate highly purified nuclei and to minimize diffusion of nuclear proteins during cell fractionation, our nuclear isolation procedure is performed in a low-salt, highly viscous sucrose–glycerol solution. In such a buffer, nuclei are the only cellular structures of sufficient density and size to sediment during the 30 min centrifugation period. During the purification of nuclei, divalent cations have been removed by EDTA and have been replaced by the polyamines spermine and spermidine (Hewish and Burgoyne, 1973), since in the presence of Mg^{2+} or Ca^{2+} chromatin is attacked by endogenous nucleases (Schibler et al., 1983). Maintaining chromatin intact may be important to guarantee its quantitative removal in the centrifugation step following the salt extraction of nuclei. Indeed, our attempts to prepare active extracts from nuclei isolated in the presence of Mg^{2+} have thus far been unsuccessful (A.-C. Pittet and U. S., unpublished observation).

We have used the protocol described in Experimental Procedures to prepare active extracts from rat liver, brain, and spleen tissue. We have also tried to apply our method to the parotid gland, but we were unable to prepare a transcriptionally competent nuclear extract from this tissue. The protein pattern of the parotid extract, as revealed by SDS–polyacrylamide gel electrophoresis, appeared similar to that of the other tissues, irrespective of whether the extract had been incubated for in vitro transcription. The template DNA, however, was rapidly degraded during incubation at 30°C, and even on ice (P. Shaw and U. S., unpublished observations). Therefore, we believe that the action of endogenous DNAase(s) rather than proteinase(s) was responsible for the failure of this extract to promote in vitro transcription.

On the basis of our experience with the tissues mentioned above, we anticipate that our procedure is applicable without modification to any tissue that can be homogenized in the highly viscous buffer and that is not a major source of digestive enzymes (as are tissues such as the parotid gland and, possibly, the pancreas).

Optimizing Parameters for In Vitro Transcription with Rat Liver Nuclear Extract

We first characterized the basic parameters of the in vitro transcription reaction. We chose assay conditions similar to those described by Parker and Topol (1984b), and,

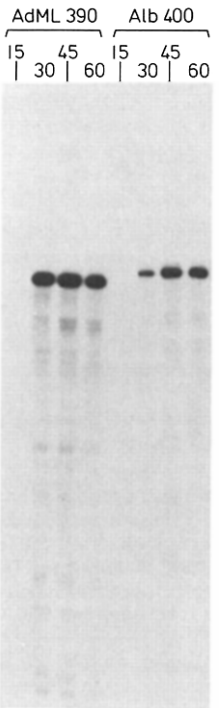

Figure 2. Effect of Extract Concentration on In Vitro Transcription of AdML390 and Alb400

A nuclear extract was prepared from rat liver tissue as described in Experimental Procedures. Transcription was performed in reactions containing 800 ng of either AdML390 (left) or Alb400 (right) and increasing amounts of the extract as a percentage of total volume in the reaction mix (the numbers indicate 15%, 30%, 45%, and 60% extract in the reaction, corresponding to 1.6, 3.2, 4.7, and 6.3 mg/ml protein). All other variables (salt concentration, etc.) were adjusted to remain constant. The assay and analysis were as described in Experimental Procedures.

using rat liver nuclear extract, we tested in vitro transcription from the three templates $p(C_2AT)_{19}$, AdML390, and Alb400 (see above) in this system. The results (Figure 1B) show that no transcripts are generated in assays containing the G-free cassette vector alone (lane 1), whereas the two promoter-containing templates give similar activities (compare lanes 2 and 3). We conclude, based on the estimated sizes of the transcription products, that both AdML390 and Alb400 are correctly initiated. Transcription from AdML390 and Alb400 is abolished in the presence of 2 μg/ml of α-amanitin, indicating that the observed transcripts are synthesized by RNA polymerase II (not shown).

The optimum concentration of extract required in the transcription reaction was determined. Figure 2 shows the results of transcription assays in which a constant amount (800 ng) of either AdML390 or Alb400 was used as template and in which the extract contributed from 15% to 60% of the total volume of the reaction. At 15% extract, no transcription is detected with either template. At 30% extract, the transcription products seen with the AdML390 promoter are already at the maximum level, whereas transcription from the Alb400 promoter is 3-fold lower than that generated with 45% extract. Unless otherwise stated, in further experiments the extract made up 45% of the

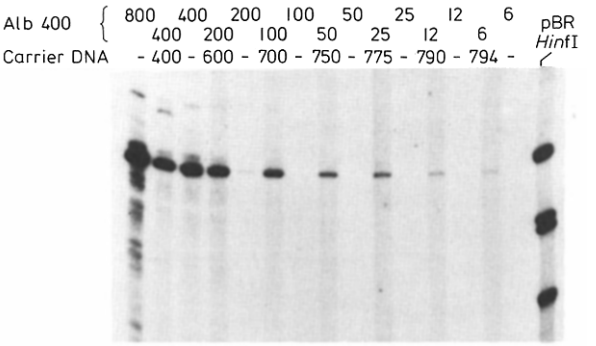

Figure 3. Template Concentration Dependence in the In Vitro Reaction

The nuclear extract preparation, assay, and analysis conditions were as described in Experimental Procedures except that the concentration of specific template (Alb400) ranged from 800 ng to 6 ng per reaction. Assays were performed using decreasing amounts of specific template, with or without the addition of high molecular weight salmon sperm DNA to a total DNA concentration of 800 ng per reaction. The protein concentration was 3.4 mg/ml in each reaction.

reaction volume; this corresponds to a protein concentration of 3–5 mg/ml.

The effect of template concentration on in vitro transcription was also examined, as illustrated in Figure 3. Reactions were performed in the presence of decreasing amounts of the plasmid Alb400, either alone or supplemented to a constant DNA concentration of 800 ng per reaction with commercial high molecular weight salmon sperm DNA. In the reaction containing specific template alone, transcription products are seen only at the two highest concentrations tested (800 ng and 400 ng per reaction), whereas in those supplemented with carrier DNA there is a detectable signal even at the lowest template concentration tested (6 ng). These results indicate that even in the presence of carrier DNA the transcription signal obtained for Alb400 is highly dependent on the template concentration. This observation is somewhat surprising, since we calculated from the amount and the specific radioactivity of the transcripts synthesized in vitro that only a minor proportion of templates (1%–2%) are used for transcription. While there are several interpretations for this apparent discrepancy, it is possible that repressor-like molecules must be depleted by an excess of template before efficient transcription is possible.

AdML390 and Alb400 linearized at either the SmaI or HindIII site in the vector polylinker directly downstream of the G-free cassette transcribe as efficiently as their circular counterparts (not shown). This suggests that the form of the template DNA, linear or circular, is not critical.

In Vitro Transcription from the Albumin Promoter Is Tissue-Specific

To examine whether in vitro transcription from the albumin promoter is tissue-specific, reactions were performed using nuclear extracts from liver, brain, and spleen. As an internal control for general transcriptional activity in the three extracts tested, AdML390 was included in each reaction. The albumin template Alb320 used in this ex-

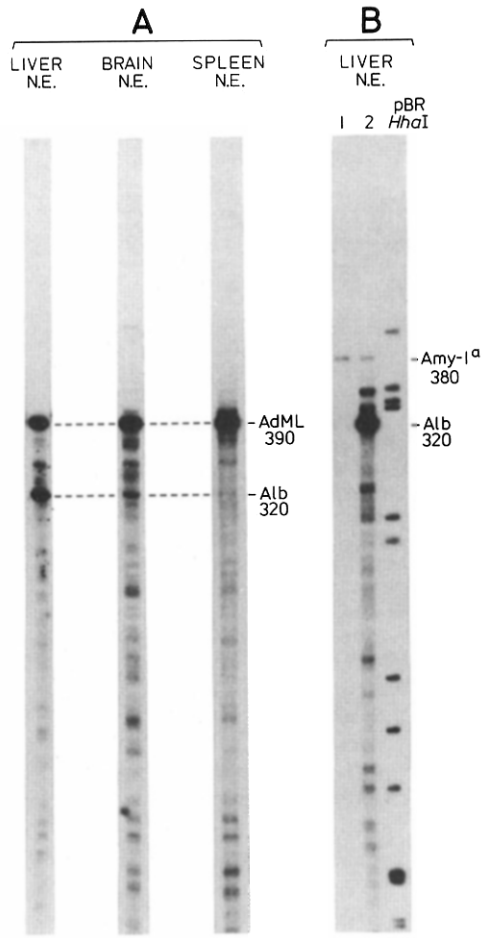

Figure 4. Promoter Utilization in Extracts Derived from Different Tissues

(A) Utilization of AdML and albumin promoters in extracts prepared from liver, brain, or spleen. Equal amounts of AdML390 and Alb320 (400 ng each) were assayed in a reaction mix containing, by volume, 45% (liver and brain) or 60% (spleen) extract prepared from these different rat tissues. The corresponding protein concentrations in the reactions were 3.8 mg/ml (liver), 3.6 mg/ml (brain), and 5.1 mg/ml (spleen). Reactions in all three tissue extracts using only AdML390 as template allowed verification that the lower band arose from transcription of Alb320. Transcripts shorter than full-length AdML390 or Alb320 arise by nucleolytic breakdown or premature termination rather than initiation at different start sites within the cassette, since the vector alone did not yield detectable transcripts (Figure 1B and unpublished observations). The transcription products were analyzed as previously described.

(B) Utilization of the *Amy-1* parotid promoter in liver extract. Lane 1, Amy380 alone (800 ng); lane 2, Amy380 plus Alb320 (400 ng each). The liver extract was the same as in (A).

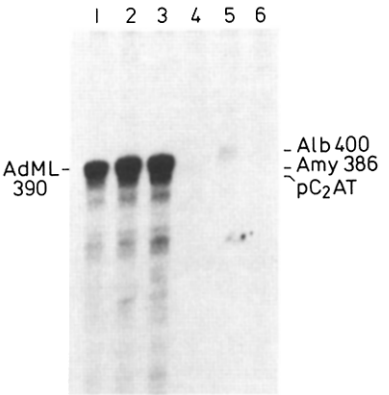

Figure 5. In Vitro Transcription of AdML390, Alb400, Amy386, and $p(C_2AT)_{19}$ in HeLa Whole-Cell Extracts

The templates AdML390, Amy386, Alb400, and $p(C_2AT)_{19}$ were cleaved to completion with the restriction endonuclease SmaI, extracted with phenol and chloroform, and incubated for in vitro transcription according to Manley et al. (1980) in a total volume of 20 μl containing 16 μl of soluble HeLa whole-cell extract. The in vitro transcription products were analyzed as described previously. The following templates were used: lane 1, AdML390 and Amy 386, 400 ng each; lane 2, AdML390 and Alb400, 400 ng each; lane 3, AdML390, 800 ng; lane 4, Amy386, 800 ng; lane 5, Alb400, 800 ng; lane 6, $p(C_2AT)_{19}$ (promoterless G-free cassette vector), 800 ng. The positions of transcripts produced by the four templates are indicated.

periment consists of the same albumin sequence (base pairs −650 to +22) present in Alb400, but it contains a shortened G-free cassette of approximately 320 nucleotides. The shorter RNA produced from this template can be easily discriminated from the one transcribed from hybrid genes containing the full-length G-free cassette. AdML390 and Alb320 were mixed in a 1:1 molar ratio and were tested in rat liver, brain, and spleen extracts. The results of this experiment are shown in Figure 4. AdML390 is active in all three extracts, demonstrating that they are all transcriptionally competent. In contrast, Alb320 is transcribed at levels comparable to that of AdML390 only in the liver nuclear extract. In the brain extract the AdML390 template is about 10-fold more active than Alb320, whereas in the spleen nuclear extract, Alb320 transcripts are barely detectable. Thus, at least qualitatively, in vitro transcription from the albumin promoter mimics the in vivo expression of the albumin gene. As mentioned in the Introduction, albumin mRNA in vivo accumulates to high levels in liver, to low levels in brain, and is undetectable in spleen.

To ensure that the preferential transcription from the albumin promoter in the liver extract does not merely reflect the permissiveness of this extract for any promoter used, we tested a promoter that in vivo is silent in liver but highly active in another tissue. For this experiment a template was constructed containing the strong parotid-specific promoter of the α-amylase gene *Amy-1* (−2,300 to −2 of *Amy-1* sequence) fused to the G-free cassette. This template (Amy380) generates a transcript approximately 380 nucleotides long. When tested in the liver extract, the Amy380 template, alone or mixed 1:1 with Alb320, is poorly transcribed (Figure 4B); the ratio of transcription signal from Alb320 to that from Amy380 is more than 30:1. Experiments with an amylase promoter extending to +6 rather than −2 (Amy386) showed the same low level of transcription (data not shown).

As mentioned above, the in vitro extracts used here have been prepared from highly purified nuclei. It could be argued, therefore, that the tissue specificity observed in our experiment could be the fortuitous result of differential loss of general transcription factors occurring during the isolation of nuclei. To examine this (rather unlikely)

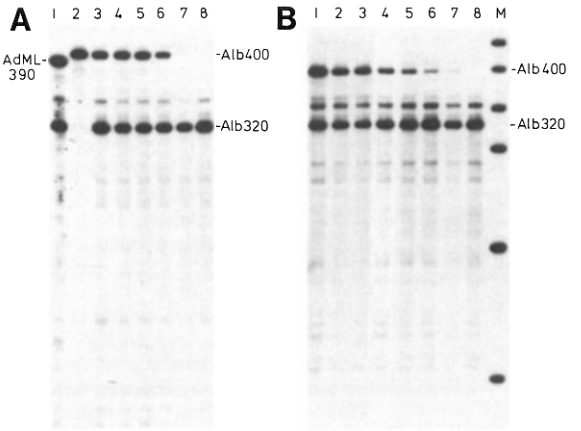

Figure 6. In Vitro Activity of Deletion Mutants of the Albumin Promoter

(A) Deletion mutants generated by restriction endonuclease cleavage. Except as indicated for lanes 1–3, 400 ng of each deletion mutant was mixed with 400 ng of Alb320. Lane 1, AdML390 and Alb320 (400 ng each); lane 2, 800 ng of Alb400; lane 3, 400 ng each of Alb400 and Alb320; lane 4, Alb400/−376 (generated by digestion with RsaI); lane 5, Alb400/−299 (HaeIII); lane 6, Alb400/−170 (AluI); lane 7, Alb400/−58 (Sau3AI). Lane 8 shows the results with 800 ng of Alb320. The protein concentration in each reaction was 3.9 mg/ml.

(B) Deletion mutants generated by treatment with the exonuclease Bal31. In each assay 400 ng of template containing a deletion was mixed with 400 ng of Alb320. The Bal31 deletions (lanes 3–8) were sequenced to determine their 5′ endpoints. Lane 1, Alb400/−299; lane 2, Alb400/−170; lane 3, Alb400/−147; lane 4, Alb400/−124; lane 5, Alb400/−94; lane 6, Alb400/−74; lane 7, Alb400/−64; lane 8, Alb400/−23. The protein concentration in each reaction was 4.9 mg/ml. Different nuclear extracts were used in (A) and (B).

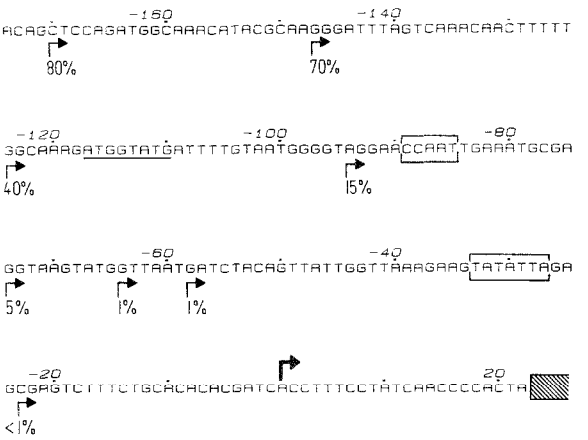

Figure 7. The Sequence of the Albumin Promoter Region

The sequence of the albumin promoter is shown from −174 to +22, where the junction with the G-free cassette occurs. The TATA box (−33) and CAAT box (−89) are indicated. The region of homology with the SV40 core enhancer is underlined. Arrows show the endpoints of all deletions, and the level of transcription of each mutant relative to the Alb320 plasmid is shown as a percentage for each deletion mutant (see Experimental Procedures). A thick arrow marks the cap site.

possibility, we performed in vitro transcriptions with several templates using whole-cell extracts from HeLa cells (Manley et al., 1980). Figure 5 shows that neither the promoter of the α-amylase gene *Amy-1* (lane 4) nor that of the albumin gene (lane 5) is significantly active in soluble whole-cell extracts from HeLa cells. As expected, the template containing the AdML promoter directs efficient synthesis of transcripts in these extracts, irrespective of whether it is offered as the only template (Figure 5, lane 3) or whether it is mixed in a 1:1 ratio with either Amy386 (Figure 5, lane 1) or Alb400 (Figure 5, lane 2). In agreement with the results obtained with nuclear extracts, the promoterless construct $p(C_2AT)_{19}$ does not yield transcripts of the expected size (380 nucleotides) in whole-cell extracts.

Efficient Liver-Specific In Vitro Transcription from the Albumin Promoter Requires Upstream Sequences

To determine the region of the albumin promoter required for efficient in vitro transcription, we generated various deletion mutants and tested their ability to direct transcription in vitro. First, a series of mutant templates was constructed by using convenient restriction endonuclease sites as described in the legend to Figure 6A. Each deletion mutant was mixed in a 1:1 molar ratio with Alb320, which served as an internal control. Quantitation by densitometry of the results shown in Figure 6A reveals that deletion mutants Alb400/−376 (lane 4; truncated at position −376) and Alb400/−299 (lane 5; truncated at position −299) retain essentially 100% of wild-type albumin promoter activity, but that a slight drop to 80% of wild-type activity may occur with the Alb400/−170 mutant (lane 6). A drastic drop (to about 1% of wild-type activity) occurs when the upstream sequences are further truncated to position −58 (Figure 6A, lane 7).

To focus more closely on the most crucial region, −170 to −58, a further series of truncated templates was created using the exonuclease Bal31. As before, these deletion mutants were mixed in a 1:1 molar ratio with Alb320 as an internal control. The products generated in vitro are shown in Figure 6B. With the mutants truncated at −170 and −147, the level of transcription is similar (80% and 70% of wild-type activity, respectively). With mutants truncated between −124 and −94 the drop is from 40% of wild-type activity to 15%, and once the CAAT box has been deleted (the mutants truncated at −74 and −64; see Figure 6B) the level of transcription drops to only 5% and 1%, respectively, of wild-type activity. The final mutant, truncated at −23, deletes the TATA box and has no detectable activity. The observation that the stepwise removal of upstream sequences results in a gradual loss of transcriptional activity may reflect the interplay of multiple factors with numerous sequence elements in the albumin promoter. Figure 7 shows the drop in level of transcription for all deletion mutants truncated between −170 and −23.

The activities of four of these deletion mutants (Alb400/−299, Alb400/−147, Alb400/−124, and Alb400/−58) were also determined in brain extract. Low-level transcription was detected with all of the mutants, although a slight drop in signal was noted between mutants Alb400/−124 and Alb400/−58 (data not shown). These observations are compatible with the finding that, in vivo, brain expresses the albumin gene at a low level.

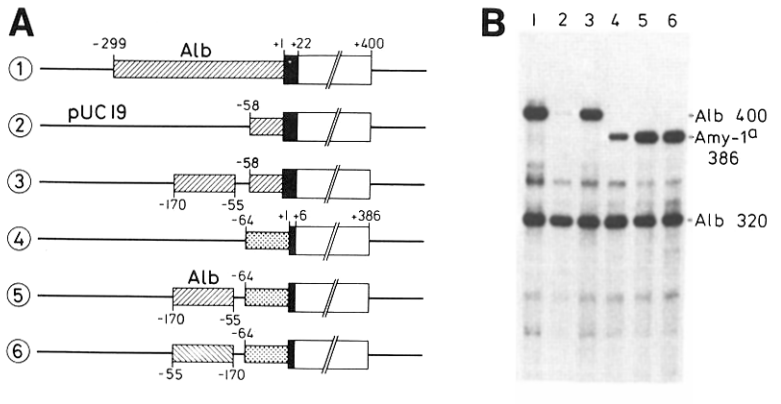

Figure 8. Insertion of Albumin Sequences Upstream of the *Amy-1* Promoter

(A) Diagram of templates. The 5′-flanking region of each gene is shown either with diagonal lines (albumin) or stipples (α-amylase). The 116 bp fragment (−170 to −55) of the albumin promoter is inserted either in the correct orientation (constructs 3 and 5) or in the opposite orientation (construct 6), 18 bp upstream of the end of the indicated promoter fragment. Construct 1, Alb400/−299; construct 2, Alb400/−58; construct 3, Alb400/−58 containing the albumin promoter insert in the correct orientation; construct 4, Amy386/−64; construct 5, Amy386/−64 containing the insert in the correct orientation; construct 6, Amy386/−64 containing the insert in the opposite orientation.

(B) In vitro transcription products. The six templates described above were used in a 1:1 ratio (400 ng each) with Alb320. The numbering of the lanes in the gel corresponds to the numbers of the constructs in (A). The protein concentration in each reaction was 4.9 mg/ml.

An Upstream Albumin Promoter Element Increases Transcription from the *Amy-1* Promoter in *Cis*

From the previous deletion analysis it is apparent that sequences upstream of −58 are required for efficient tissue-specific in vitro transcription from the albumin promoter. We then tested whether an upstream segment of albumin DNA could increase transcription from the *Amy-1* promoter, which by itself is considerably less active in liver extracts than is the albumin promoter. To this end, a 116 bp albumin promoter fragment (−170 to −55) was inserted in both orientations at the EcoRI site of a plasmid (Amy386/−64) containing a truncated *Amy-1* promoter (−64 to +6) fused to the G-free cassette. In this plasmid, the albumin element mentioned above is separated from *Amy-1* position −64 by 18 bp of the pUC19 vector sequence. Figure 8A illustrates these constructs. As shown in Figure 8B, when inserted upstream of residue −58 of albumin, this 116 nucleotide sequence restores in vitro transcription from the albumin promoter (compare lanes 1 and 3). Lanes 5 and 6 in Figure 8B show the results obtained using the templates bearing the albumin–α-amylase hybrid promoters. Insertion of the 116 bp albumin promoter fragment considerably increases transcription from the α-amylase promoter mutant truncated at −64. The Alb320 to Amy386/−64 transcription ratio is 5:1, but with the albumin fragment inserted in either orientation upstream of position −64 of *Amy-1* the ratio becomes approximately 1:1. Interestingly, the *Amy-1* promoter truncated at position −64 is at least 5-fold more active in the liver extract than is its counterpart containing 2.3 kb of *Amy-1* 5′-flanking sequence (see Figure 4B). This result is compatible with transfection experiments performed in our laboratory that revealed a negative regulatory element between positions −500 and −300 of the parotid-specific *Amy-1* promoter (R. Walter and U. S., unpublished observations).

The Albumin Promoter Is Active in Mixed Extracts

The requirement for albumin upstream sequences for efficient in vitro transcription suggests that positive transcription factors are present in the liver extract. This does not, however, exclude the possibility that negative *cis*-acting elements and *trans*-acting factors are involved in establishing differential albumin transcription. Indeed, as pointed out above, the strong dependence of the transcription signal on the concentration of Alb400 template (Figure 3) is compatible with the existence of repressors. Thus, the poor transcriptional activity of the albumin promoter in brain and spleen extracts could be due either to a deficiency of activating factors or to an excess of repressors in these extracts. To discriminate between these two possibilities, a transcription assay was performed in which brain and liver extracts that are similarly active for AdML390 transcription were mixed in a 1:1 molar ratio. Figure 9 shows that the mixed extract (lane 3) is almost as efficient as the liver extract alone (lane 1) in promoting transcription from the albumin promoter. It appears likely, therefore, that the failure of brain extracts to promote efficient albumin in vitro transcription is due to an insufficient concentration (and/or activity) of positive factors rather than a surplus of negative factors.

Discussion

We report here the preparation of transcriptionally active nuclear extracts from rat liver, brain, and spleen. In these soluble extracts, the promoter of the mouse albumin gene behaves, at least qualitatively, as it does in vivo. It promotes efficient transcription in the presence of nuclear proteins from liver, is only slightly active in brain extract, and is almost silent in spleen extract. The preferential utilization of the albumin promoter in an extract from liver nuclei is not due to a general transcriptional permissiveness of this extract since it does not promote significant

Figure 9. In Vitro Transcription from the Albumin Promoter in Mixed Liver and Brain Extracts

A 1:1 mixture of AdML390 and Alb320 (400 ng each) was transcribed in vitro in liver extract (lane 1), brain extract (lane 2), or mixed extract containing 50% liver and 50% brain nuclear proteins (lane 3). The in vitro transcription reactions contained 4.4 mg/ml of liver extract, 4 mg/ml of brain extract, and 4.2 mg/ml of mixed extract.

transcription from the strong parotid-specific promoter of the mouse α-amylase gene *Amy-1* (Schibler et al., 1983).

Sequences located between 170 and 55 nucleotides upstream of the cap site are required for efficient liver-specific in vitro transcription from the albumin promoter. Stepwise removal of sequences within this region results in a gradual loss of activity. Both of these findings are in agreement with results obtained in vivo. Thus, in transfection studies Ott et al. (1984) observed that tissue-specific regulatory elements reside within the immediate 5′-flanking region of the albumin gene. Promoter deletion analysis recently performed by the same group (J. M. Heard, P. Herbomel, M.-O. Ott, A. Mottura-Rollier, M. Weiss, and M. Yaniv, submitted for publication) revealed gradual loss of reporter gene (chloramphenicol acetyl transferase) activity in transfected cells when sequences downstream of residue −156 were deleted stepwise to −54. While these in vivo studies are qualitatively in agreement with our in vitro results, the decreases in activity seen in vivo are larger than the corresponding drops observed in our in vitro transcription experiments. The stepwise decrease in promoter activity that we observe as upstream sequences are deleted suggests the involvement of multiple transcription elements. This speculation is supported by DNAase I footprint analysis that shows at least three distinct binding sites for liver nuclear proteins within this region (S. Lichtsteiner and U. S., unpublished observations). Evidence from other systems also implicates multiple elements in the control of tissue-specific gene expression (see, for example, Grosschedl and Baltimore, 1985; Ohlsson and Edlund, 1986).

The sequences from −170 to −55, when inserted in either orientation upstream of residue −64 of *Amy-1*, augment in vitro transcription to the level observed for the albumin promoter. This ability to act independently of orientation has been observed for many promoter and enhancer elements (reviewed by Serfling et al., 1985). Enhancer sequences, in particular, augment transcription even when located far away and/or downstream of the cap site (Banerji et al., 1981; Moreau et al., 1981). While we have not yet evaluated whether the *cis*-acting albumin element can also exert its effect over large distances or downstream of the start site, our experiments indicate that small changes in distance from the transcription initiation site do not seem to perturb its activity. A promoter with an 18 nucleotide insertion and the albumin–α-amylase hybrid promoter are as efficient as the albumin wild-type promoter in directing in vitro transcription. It may be noteworthy in this context that the sequence ATGGTATG, located between positions −117 and −110 of the albumin promoter element (Figure 7), shares seven out of eight nucleotides with the SV40 enhancer core sequence GTGG$^{AAA}_{TTT}$G (Weiher et al., 1983; Khoury and Gruss, 1983).

Several results indicate that the tissue-specific in vitro transcription from the albumin promoter that we have observed in our experiments is controlled by the action of positive transcription factors present in the liver nuclear extract. Our reasoning is based on two findings: first, upstream sequences are required for efficient transcription; and second, in mixing experiments the activity of the liver extract is not repressed by the brain extract. There is, however, evidence from experiments with somatic cells that negative regulatory mechanisms may also influence the specificity of albumin transcription. If Chinese hamster fibroblasts are fused to rat hepatoma cells, albumin expression is extinguished in the resulting hybrid cell lines (Weiss et al., 1975). More recently, it has been shown that in microcell hybrids between rat hepatoma cells and mouse fibroblasts, albumin extinction is mediated by one particular chromosome of the mouse fibroblast parent (Petit et al., 1986). It is not yet clear whether the factor(s) producing extinction are fibroblast-specific or whether this cell fusion merely leads to an imbalance between positive factors (which normally would be dominant over repressors in hepatocytes) and ubiquitous negative factors. The latter possibility implies that repressors are also present in liver. This would explain the requirement for a high concentration of template, which we estimated to be 5,700 templates per nucleus-equivalent of extractable protein present in our in vitro extracts. Alternatively, a large number of templates may be needed to search successfully for a limited number of critical positive regulatory molecules.

We feel confident that the in vitro transcription system described here will be a valuable tool in the identification and characterization of positive and negative *trans*-acting tissue-specific transcription factors.

Experimental Procedures

Plasmid Constructions

The $p(C_2AT)_{19}$ and $pML(C_2AT)_{19}$ plasmids (the latter referred to herein as AdML390) were a generous gift of M. Sawadogo and R. G. Roeder. Alb400 was constructed by inserting the gel-isolated 672 bp MaeI fragment from the mouse albumin gene (base pairs −650 to +22) into the SstI site of $p(C_2AT)_{19}$ after the ends of both insert and vector had been blunted with T4 DNA polymerase. Alb320 was constructed by cleaving Alb400 with SmaI, incubating the linearized DNA with exonuclease Bal31, and religating the deleted molecules with T4 DNA ligase. Several clones were tested in an in vitro transcription assay using liver nuclear extract, and one was selected that produced an RNA approximately 320 nucleotides long.

Albumin promoter deletion mutants

To construct the series of deletion mutants based on restriction endonuclease sites, double enzymatic digestions of Alb400 were performed using SmaI and either RsaI (recognition site −378), HaeIII (−301), or AluI (−172). The mutant truncated at −58 was derived from a partial Sau3AI digestion of the AluI–SmaI fragment, since there is another Sau3AI site at position −4 in the albumin promoter. In each case, the appropriate DNA fragments were isolated by electrophoresis and then subcloned into the SmaI-cleaved vector pUC19.

The Bal31-derived deletions were constructed by cleaving the Alb400/−170 plasmid with EcoRI and then incubating the linear DNA with Bal31. DNA was removed from the reaction at various times and was digested with SmaI. Blunt-ended DNA fragments of the appropriate size range were isolated by electrophoresis and were then cloned into SmaI-cleaved pUC19. Two mutants were selected for sequence analysis to determine the 5′ deletion boundary. One of them, Alb400/−124, was used to generate additional deletions by the same procedure. The 5′ endpoints of these further deletions (Alb400/−94, Alb400/−74, and Alb400/−64) were also determined by sequencing. The deletion mutant Alb400/−23 was generated in the same manner but using Alb400/−58 as starting material.

Amylase promoter constructs

Amy380 and Amy386 each contain a 2.3 kb insert from the parotid promoter of *Amy-1* cloned upstream of the G-free cassette. They were constructed by isolating by electrophoresis a 2.3 kb MaeII fragment (−2,300 to +9 of *Amy-1*) and then treating this fragment with nuclease S1 to remove the 5′ overhang and thus the G residue present in the MaeII site that would be incorporated in a fill-in reaction. The fragments were blunted with T4 DNA polymerase and then cloned into the blunted SstI site of $p(C_2AT)_{19}$. Sequence analysis revealed the 3′ junction with the G-free cassette to be position −2 in the case of Amy380 and +6 in the case of Amy386. The Amy386/−64 deletion mutant was constructed by cloning the BalI–SmaI fragment (−64 of *Amy-1* to the end of the G-free cassette) of Amy386 into SmaI-cleaved pUC19.

Insertion mutants

The AluI–Sau3AI fragment (−170 to −55) of the albumin promoter was isolated by electrophoresis, filled in with T4 DNA polymerase, and inserted into the blunted EcoRI site of Alb400/−58. A plasmid containing the insertion in the positive orientation was isolated. The same fragment was also inserted into the blunted EcoRI site of Amy386/−64, and clones containing the insert in both orientations were selected for in vitro analysis.

DNA Manipulations

Plasmids were purified from Escherichia coli strain JM101 by the method of Sahli et al. (1985) except that 5 ml cultures were used and all other volumes were doubled. The final precipitate was dissolved in 80 μl of 10 mM Tris–HCl (pH 8), 2 mM EDTA containing 100 μg/ml of RNAase A and 100 units/ml of RNAase T1, and was incubated at 37°C for 30 min. The reaction was terminated by the addition of 10 μl each of 10% SDS and 0.5 M EDTA (pH 8), and the entire sample applied to a Pasteur-pipette column of Biogel A-1.5m (Bio-Rad) preequilibrated in 10 mM Tris–HCl (pH 8), 1 mM EDTA. Fractions of 100 μl were collected, and those containing the plasmid DNA (monitored by ethidium bromide fluorescence) were pooled and made 0.2% in SDS and 0.5 M in NaCl. After three extractions with chloropane (phenol and chloroform [1:1] saturated with 10 mM sodium acetate [pH 6.0], 0.1 M NaCl, 1 mM EDTA), the DNA was precipitated with two volumes of ethanol and was resuspended at a concentration of 800 μg/ml in 10 mM Tris–HCl (pH 8), 0.1 mM EDTA. We usually obtain between 20 and 40 μg of plasmid DNA per 5 ml of bacterial culture using this method. DNA prepared in this fashion was also used for sequence analysis (Chen and Seeburg, 1985). When more material was required, a 10-fold scaleup of this procedure was used.

All other DNA manipulations for cloning followed the procedures outlined by Maniatis et al. (1982).

Extract Preparation

All manipulations were performed in the cold, and all solutions, tubes, and centrifuges were chilled to 0°C. When included, phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT) were added to the buffers just prior to use. Minced tissue (10–15 g of liver and brain, 2 g of spleen) was brought up to 30 ml with homogenization buffer (10 mM Hepes [pH 7.6], 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol) and was homogenized using a motor-driven 30 ml Teflon–glass homogenizer until more than 90% of the cells were broken. The homogenate was diluted to 85 ml with homogenization buffer, layered in three 27 ml aliquots over three 10 ml cushions of the same buffer, and centrifuged at 24,000 rpm for 30 min at −2°C in an SW27 rotor. The combined nuclear pellets were resuspended in 50 ml of a 9:1 (v/v) mixture of homogenization buffer and glycerol, again using a Teflon–glass homogenizer. This homogenate was layered over two 10 ml cushions as described above, and centrifuged under the same conditions. The pelleted nuclei were resuspended in 20 ml of nuclear lysis buffer (10 mM Hepes [pH 7.6], 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol) using an all-glass Dounce homogenizer (pestle A). An aliquot was diluted 1:20 in 0.5% SDS, and the absorbance at 260 nm was measured. The nuclear suspension was diluted to approximately 10 A_{260} units per ml and was extracted essentially as described by Parker and Topol (1984a). One-tenth volume of 4 M $(NH_4)_2SO_4$ (brought to pH 7.9 with NaOH) was added dropwise, and the extract was gently shaken for 30 min. The viscous lysate was then centrifuged at 35,000 rpm for 60 min in a Ti60 rotor (0°C) to pellet chromatin. Solid $(NH_4)_2SO_4$ (0.3 g/ml) was added to the supernatant and was slowly dissolved (over approximately 15 min). After an additional 20–40 min on ice, the precipitated proteins were sedimented by a 25 min centrifugation at 35,000 rpm in a Ti60 rotor. The protein pellet was either stored in ice overnight or was directly resuspended in dialysis buffer at 1 ml per 20 A_{260} units of nuclear lysate (see above) for liver and brain or 1 ml per 40 A_{260} units of nuclear lysate for spleen. (Dialysis buffer consists of 25 mM Hepes [pH 7.6], 40 mM KCl, 0.1 mM EDTA, 1 mM DDT, and 10% glycerol.) The protein extract was dialyzed twice for 2 hr, each time against 250 ml of the same buffer. During dialysis, a precipitate forms that at the end of the dialysis was removed by centrifugation in a microfuge for 5 min. An aliquot was diluted 40-fold in distilled water, and the protein concentration was determined according to Kalb and Bernlohr (1977). We normally obtain approximately 10 mg of nuclear protein per adult rat liver. The protein extract was frozen in small aliquots and was stored in liquid nitrogen. Extracts stored in liquid nitrogen remained active for at least 6 months, whereas extracts stored at −70°C lost some activity over this same time period. Once thawed, the extracts were not refrozen.

Whole-cell extracts from exponentially growing HeLa cells were prepared exactly as described by Manley et al. (1980).

In Vitro Transcription Assays

Transcription reactions (20 μl) contained 40 μg/ml of circular DNA template and 3–5 mg/ml nuclear protein extract in a buffer containing 25 mM Hepes (pH 7.6), 50 mM KCl, 6 mM $MgCl_2$, 0.6 mM each of ATP and CTP, 35 μM UTP, 7 μCi [α-^{32}P]UTP (Amersham; 400 Ci/mmol), 0.1 mM 3′-O-methyl GTP (P-L Biochemicals), 12% glycerol, and 1 μl RNAsin (approximately 30 units; Promega Biotec). EDTA at 0.045–0.06 mM and DTT at 0.45–0.6 mM were contributed by the extract. After 45 min of incubation at 30°C (a time course indicated that no further RNA synthesis occurred after 45 min), the reactions were terminated by the addition of 380 μl of stop buffer (50 mM Tris–HCl [pH 7.5], 1% SDS, 5 mM EDTA, 25 μg/ml tRNA) and were extracted three times with chloropane (see above). The RNA was precipitated by the addition of 40 μl of 3 M sodium acetate (pH 4.8) and 880 μl of ethanol. The RNA pellets were rinsed with 70% ethanol, air-dried, and resuspended in 10 μl of loading

mix (80% formamide, 0.01% xylene cyanol, and 0.01% bromophenol blue in 1× TBE), and 4 μl aliquots were then loaded on a 4% polyacrylamide–7 M urea sequencing gel. Autoradiography was carried out at −80°C with intensifying screens. Most autoradiograms were quantitated by densitometry. The values noted in the text and in Figure 7 are the averages of at least two scans from two different assays. In some experiments the regions of the polyacrylamide gels containing the full-length transcripts were excised and then counted in Aquasol-2 (New England Nuclear) in a liquid scintillation counter. From the specific radioactivity of the UTP in the reaction and the amount of radioactivity associated with the full-length transcripts, we estimated that approximately 2–6 fmol of AdML RNA was synthesized per 0.3 pmol of template DNA. We have found some variability in the relative transcription efficiencies for different promoters among different extracts. Thus, the ratio of AdML to albumin transcripts has varied between 2.5 and 1 in liver extracts and between 20 and 9 in brain extracts.

In vitro transcriptions using HeLa cell extracts were performed according to the procedure of Manley et al. (1980) in 20 μl reactions using 16 μl of whole-cell extract and 800 ng of DNA template. GTP was replaced by 900 μM of the chain terminator 3′-O-methyl GTP. Experiments with both linearized (see legend to Figure 5) and circular templates were performed. As observed with extracts from purified nuclei, the relative transcription efficiencies from the different promoters were similar in Manley extracts irrespective of the form (circular or linear) of DNA templates. Because of the presence of endogenous GTP in whole-cell extracts, a considerable proportion of AdML390 transcripts (which were the only clearly detectable transcripts) extended beyond the SmaI site flanking the G-free cassette, in spite of the high concentration of chain terminator used, when circular DNA was offered. Therefore, only the results obtained with linearized templates are presented.

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