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Repression of the Albumin Gene in Novikoff Hepatoma Cells

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Novikoff hepatoma cells have lost their capacity to synthesize albumin. As a first approach to study the mechanisms underlying this event, in vitro translation in a reticulocyte system was performed using total polyadenylated mRNA from rat liver and Novikoff hepatoma cells. Immunoprecipitation of the in vitro translation products with albumin-specific antibody revealed a total lack of albumin synthesis in Novikoff hepatoma, suggesting the absence of functional albumin mRNA in these cells. Titration experiments using as probe albumin cDNA cloned in pBR322 plasmid demonstrated the absence of albumin-specific sequences in both polysomal and nuclear polyadenylated and total RNA from Novikoff cells. This albumin recombinant plasmid was obtained by screening a rat liver cDNA library with albumin [^{32}P]cDNA reverse transcribed from immunoprecipitated mRNA. The presence of an albumin-specific gene insert was documented with translation assays as well as by restriction mapping. Repression of the albumin gene at the transcriptional level was further demonstrated by RNA blotting experiments using the cloned albumin cDNA probe. Genomic DNA blots using the cloned albumin cDNA as probe did not reveal any large-scale deletions, insertions, or rearrangements in the albumin gene, suggesting that the processes involved in the suppression of albumin mRNA synthesis do not involve extensive genomic rearrangements.

Novikoff hepatoma cells are characterized by restricted gene expression when compared to normal rat liver, displaying a lower kinetic complexity in their mRNA, and lacking a great proportion of the sequences found in the abundant mRNA populations of normal cells (5). That probably represents transcriptional control, as can be further demonstrated first from the existence of reduced complexity in the hepatoma heterogeneous nuclear RNA (hnRNA), compared to that of normal liver, as well as from cross-hybridization reactions which demonstrated that about 30% by weight of the liver hnRNA sequences are absent in Novikoff hepatoma nuclei (5). Nevertheless, the above suggestion cannot easily be proved true, using only total polyadenylated RNA populations. Specific probes would enormously facilitate the investigation of transcriptional control of gene expression in hepatoma cells as well as any other related processes.

Preliminary experiments in our laboratory using immunofluorescence and immunoprecipitation techniques demonstrated that Novikoff cells have lost their capacity to synthesize albumin, the most abundant protein in liver cells (7, 23, 25). In a similar way, it has been shown that the synthesis of albumin is reduced in some hepatomas (3, 26, 29, 31, 36), in inverse relation to the synthesis of alpha-fetoprotein (26). It is thus of great interest to study the molecular mechanisms involved in albumin gene expression in Novikoff hepatoma cells.

In the present work we have investigated possible mechanisms for the alteration of albumin gene expression in Novikoff cells. Using either in vitro translation or hybridization to albumin-specific cDNA sequences as an assay, we have quantitated the levels of albumin-specific RNA in different RNA fractions of normal rat liver and Novikoff cells. Our results strongly indicate that the albumin gene is not transcribed in Novikoff hepatoma cells, despite the indications derived from genomic blots that the gene is possibly neither deleted nor rearranged at least in large scale.

MATERIALS AND METHODS

Animals and tumors. Male Sprague-Dawley rats (150 to 200 g), used throughout, were fed ad libitum and kept in temprature-controlled rooms with alternating 12-h cycles of light and dark. Novikoff hepatoma tumors were obtained by transplanting 0.2 ml of ascites cells subcutaneously into the back of the animals 7 to 8 days before the experiments. The tumors used were carefully dissected to remove connective and necrotic tissues.

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Isolation of polysomes. Polysomes were prepared from both rat liver and Novikoff hepatoma by a modification of the method described by Sippel et al. (34). Fresh tissue was homogenized in 6 volumes of 140 mM sucrose and 2 mg of heparin per ml in polysomal buffer (25 mM Tris-hydrochloride [pH 7.5], 25 mM NaCl, 5 mM $MgCl_2$), and then Triton X-100 and sodium deoxycholate were added successively to a final concentration of 0.5%. A supernatant, prepared by centrifugation at 13,000 rpm for 10 min, was layered on a discontinuous sucrose gradient of 4 ml of 2.5 M sucrose, 7 ml of 1 M sucrose, and 1 ml of 0.5 M sucrose in polysomal buffer containing 500 μg of heparin per ml. After centrifugation for 4 h at 25,000 rpm, polysomes were recovered from the interphase between the 1 M and 2.5 M sucrose layers.

To minimize possible contamination of the polysomes with nuclear ribonucleoprotein particles, the polysomes were fractionated by preparative sucrose gradients. Polysomes greater than trimers were pooled and used for RNA isolation.

Isolation of nuclei. Nuclei were prepared from both normal rat liver and Novikoff hepatoma by the citric acid method. The fresh tissues were homogenized in 5% (wt/vol) citric acid and centrifuged at 1,000 rpm for 5 min. The pellets were suspended in 0.25 M sucrose in 1.5% (wt/vol) citric acid, homogenized, layered over equal volumes of 0.88 M sucrose containing 1.5% citric acid, and centrifuged at 2,000 rpm for 5 min. This procedure was repeated until no cytoplasmic contamination could be detected under the phase-contrast microscope.

Isolation of polysomal and nuclear RNA. Polysomal RNA was extracted with proteinase K. The polysomes were dissolved in 0.1 M sucrose–50 mM Tris-hydrochloride (pH 7.4)–0.1 M NaCl–10 mM $MgCl_2$–1% sodium dodecyl sulfate (SDS) and incubated with 100 μg of proteinase K per ml at 30°C for 2 h. The solution was then extracted twice with 1 volume of chloroform, and the RNA was precipitated with ethanol and washed.

For the isolation of nuclear RNA, the nuclei were gently homogenized at 0°C with a Duall homogenizer in 4 to 8 volumes of Sarkosyl buffer (0.1 M Tris [pH 8.0]–4% [wt/vol] Sarkosyl). One gram of solid CsCl per ml was added, and the homogenate (2.7 ml) was layered onto 0.8 ml of 5.7 M CsCl–0.1 M EDTA cushion in a polyallomer tube and centrifuged in an International SB-405 rotor at 40,000 rpm for 12 h at 25°C. After centrifugation, the DNA which banded at the interphase of the two CsCl solutions was removed with a Pasteur pipette, and the RNA in the clear pellet was dissolved in 2 ml of sterile water, precipitated twice with ethanol, and then desalted by filtration through a Bio-Gel P-4 column (9).

Total cellular RNA was prepared also by CsCl centrifugation, as described above, starting from whole tissue.

Oligodeoxythymidylate-cellulose chromatography. Isolation of polyadenylated RNA was performed by the method of Aviv and Leder (1). rRNA contamination was measured as described previously (5).

In vitro translation and immunoprecipitation. The reticulocyte system was prepared as described by Pelham and Jackson (22).

For protein synthesis, 10 μl of the preincubated lysate, 1 μl of [^{35}S]methionine (1,000 to 1,300 Ci/mmol, 5 to 6 μCi/μl), and 1 μl (1 μg/μl) of mRNA were incubated at 30°C for 40 min.

Immunoprecipitation of the in vitro translation products with antialbumin was performed by absorbance of antigen-antibody complex on *Staphylococcus aureus* bacteria mainly by a modification of the method described by Jonnson and Kronvall (13) and Kessler (14). Before use, the bacteria obtained in a 100-mg/ml suspension in 20 mM Tris-hydrochloride [pH 7.4]–0.1% SDS were washed three to four times at 4°C with a buffer containing 20 mM Tris [pH 7.4]–0.1% SDS–1% NaCl–1% deoxycholate-Triton-X (TSNDT buffer) and resuspended in the same volume of this buffer. The in vitro translation mixture (12 μl) was diluted 10-fold with the TSNDT buffer and centrifuged at 12,000 rpm for 30 min at 4°C. Fifty-microgram antibodies were added to the above supernatant, mixed, and incubated at 4°C for 1 h. A 200-μg amount of bacteria was added, and the mixture was shaken for one more hour in the cold room. After incubation, the bacteria adsorbent was centrifuged through a 100-μl sucrose cushion (1 M sucrose–10 mM methionine or leucine–20 mM EDTA–1% deoxycholate-Triton-X–0.14 M NaCl–20 mM Tris [pH 7.5]) at 10,000 rpm for 15 min. The pellet was washed two to three times through the same sucrose cushion and then dissolved in sample SDS buffer which had been heated at 95°C for 5 min and centrifuged at 10,000 rpm for 10 min, and the supernatant was applied to the gels.

Characterization of the in vitro translation and immunoprecipitation products was done by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (15).

Isolation of chimeric plasmids carrying albumin mRNA sequences. The rat liver cDNA library used (a kind gift of W. Roewekamp and W. Schmidt) was prepared from total polyadenylated polysomal RNA. Double-stranded cDNA was inserted in the *PstI* site of the pBR322 plasmid by the polydeoxyguanylate-polydeoxycytidylate tailing method described by Roewekamp and Firtel (24). Transformation was accomplished in X1776 *Escherichia coli* cells. (All of the recombinant manipulations were done according to the rules published by the Department of Health of the Federal Republic of Germany.) The library was screened by the Grunstein and Hogness method (10) using [^{32}P]cDNA transcribed from mRNA isolated by immunoprecipitation of albumin polysomes as described previously (11). Plasmid DNA was isolated by the method of Clewell and Helinski (6).

For the purpose of indentification of the albumin recombinant clones, translation arrest experiments were done as described by Paterson and Bishop (21).

Labeling of albumin insert by nick translation. The nick translation procedure used was based on that of Balmain and Birnie (4). The reaction was done in a 25-μl volume containing 50 mM Tris-hydrochloride [pH 7.5], 5 mM $MgCl_2$, 4 μg of β-mercaptoethanol per ml, 4 μM dATP, 4 μM dTTP, 50 μCi of [^{32}P]dGTP (400 Ci/mmol), 50 μCi of [^{32}P]dCTP (400 Ci/mmol) (125 pmol each), 0.05 ng of DNase I (freshly diluted from 10-μg/ml stock solution), 0.2 μg of albumin insert DNA, and 2 U of *E. coli* DNA polymerase. The mixture was incubated at 15°C for 90 min, extracted with phenol, and chromatographed over Sephadex G-50 column.

Preparation of single-stranded albumin-cloned probe. A double-stranded albumin sequence was excised

from the albumin clone by digestion with *Pst*I. This albumin sequence was nick-translated with [^{32}P]dNTPs and hybridized to Rot 1 with rat liver polysomal polyadenylate [poly(A)]-containing RNA containing about 100-fold excess of albumin mRNA in 2× SSC at 37°C. The DNA/mRNA hybrids were bound to oligodeoxythymidylate-cellulose, and after washing exhaustively the single-stranded albumin sequences were eluted with 50 mM NaOH. The remaining RNA was destroyed by further treatment with 0.3 M NaOH, and, after neutralization, 50 μg of tRNA was added and the DNA was passed through Sephadex G-50 and precipitated.

Titration of RNAs with albumin cDNA. The content in albumin sequences of different RNA fractions of rat liver and Novikoff hepatoma was determined by titration hybridization reactions as described by Young et al. (39) using the single-stranded albumin probe. Fixed amounts of albumin cDNA were hybridized with increasing RNA concentrations, and the hybrids were analyzed with S1 nuclease as previously described (11).

Genome blots. Rat liver and Novikoff hepatoma high-molecular-weight DNA, isolated by a modification of the method described by Firtel and Bonner (8), was digested twice with a fivefold excess of *Eco*RI and *Hin*dIII restriction endonucleases, and the resulting fragments were separated by electrophoresis on 0.8% agarose gel (in 4 mM Tris–4 mM sodium acetate–1 mM EDTA [pH 7.9]). The separated restriction DNA fragments were transferred to nitrocellulose using the procedure of Southern (35) as modified by Wahl et al. (37), and hybridization to nick-translated radioactive probe was then performed as described by Wahl et al. (37).

RNA blots (Northern blots). For the purpose of RNA blotting, the different RNA samples were separated by electrophoresis on 1.3% agarose horizontal slab gels in RB buffer (20 mM morpholinepropanesulfonic acid–5 mM sodium acetate–1 mM EDTA [pH 7.0]) and 2.2 M formaldehyde. Samples were adjusted to 50% formamide–2.2. M formaldehyde–RB buffer, left about 5 min at 60°C, cooled, and applied to the agarose. Transfer to nitrocellulose filter and hybridization were essentially done by the method used for DNA blots.

RESULTS

Translational capacity of mRNA from rat liver and Novikoff hepatoma cells. As a first approach to assess transcriptional control of albumin synthesis in the tumor cells, mRNAs were isolated from both cell types and assayed for their translational activity in a cell-free protein-synthesizing system derived from rabbit reticulocytes. Immunoprecipitation of the in vitro translation products with antialbumin was analyzed by SDS electrophoresis (Fig. 1). As it is clearly shown, whereas in normal liver an evident albumin-specific band could be demonstrated, no similar polypeptide is observed in the translational products derived from Novikoff cells. These results show that the quantity of albumin synthesized by the translation assay directed with Novikoff mRNA is either too small to be demonstrated with the antibody technique or that albumin is not synthesized at all.

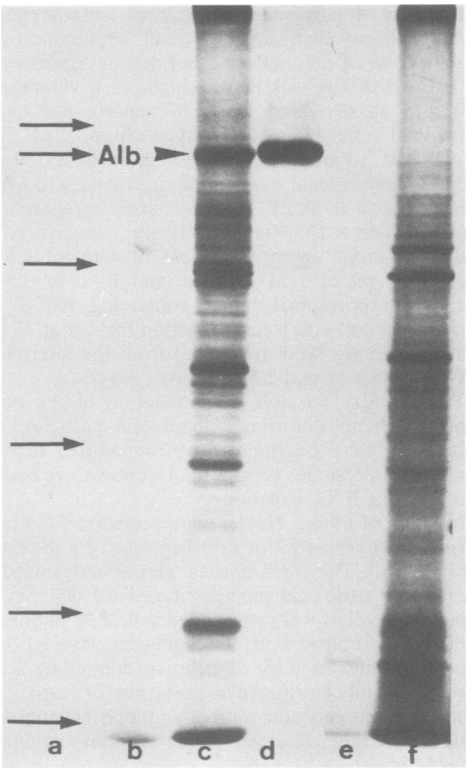

FIG. 1. Immunoprecipitation of the in vitro translation products of rat liver and Novikoff hepatoma mRNA with antialbumin. Polysomal poly(A)-containing RNAs were translated in vitro using reticulocyte cell-free system, and a part of the synthesized polypeptides were immunoprecipitated with antialbumin as described in the experimental procedures. Total and immunoprecipitated ^{35}S-labeled products were subjected to electrophoresis on SDS-10% polyacrylamide gels. (a) Markers: Phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), lysozyme (14,300). (b) Endogenous activity of reticulocyte system. (c) Liver mRNA products. (d) Immunoprecipitation of liver mRNA products. (e) Immunoprecipitation of Novikoff hepatoma mRNA products. (f) Novikoff hepatoma mRNA products.

Identification and characterization of recombinant albumin plasmids. The rat liver cDNA library was screened with [^{32}P]cDNA synthesized from immunoprecipitated albumin polysomes, and the positive clones were prepared for further characterization.

Two different methods were used to identify recombinants carrying albumin sequences. The first one consisted of a simple restriction site

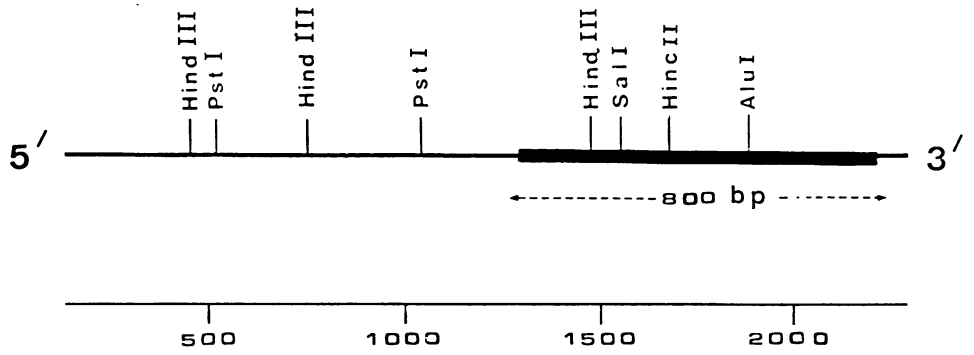

FIG. 2. Restriction site map of the albumin insert (800 base pairs). The *Hin*dIII and *Sal*I restriction endonuclease cleavage sites are shown in relation to the known albumin cDNA restriction map reported by Sargent et al. (31). The present albumin insert is shown by the brighter strong black line fitting exactly at this position.

mapping of the insert sequence, taking advantage of the known restriction site map of the albumin cDNA also cloned in pBR322 by Sargent et al. (28). Figure 2 shows the analysis of such digests of one of the identified clones after agarose electrophoresis compared to the pattern published by Sargent et al. (28).

In a second approach, translation arrest experiments were done. The nicked recombinant plasmid was hybridized with liver poly(A)-containing RNA, and in vitro translation followed either directly or after heating at 95°C. Immunoprecipitation of the revealed polypeptides with antialbumin is shown in Fig. 3, where it is clearly demonstrated that the clones used blocked the synthesis of albumin, which is released only after heating. Finally, hybridization selection experiments (data not shown) were done as a further confirmation. It is thus obvious that the analyzed clone carries albumin recombinant plasmid. The inserted sequence has a length of 800 base pairs and is oriented at the 3' end site of the albumin mRNA.

Titrations of normal rat liver and Novikoff hepatoma RNAs using single-stranded albumin cloned probe. The above results obtained from in vitro translation and immunoprecipitation experiments suggested that the inability of the Novikoff hepatoma cells to synthesize albumin was related to the absence of the corresponding functional mRNA from the polysomes of these cells. However, these results are no conclusive proof, since a variety of factors or conditions may influence in vitro translation of eucaryotic mRNAs (32). An albumin-specific probe is thus necessary to detect the presence of albumin sequences in these cells. Furthermore, in vitro translation provides no information as to whether the disappearance of the polysomal albumin mRNA molecules in Novikoff hepatoma cells, if true, is due to transcriptional control or to modulation at the level of processing or transport.

Preliminary titration experiments (data not shown) in this laboratory, using a cDNA probe reverse transcribed from immunoprecipitated albumin mRNA (11), have shown that there is

FIG. 3. Immunoprecipitation with antialbumin of the cell-free products of translation arrest. Liver polysomal poly(A)-containing RNA was hybridized to "albumin" recombinant pBR322 plasmid and then translated before (a) and after (b) heat melting of the hybrid. Immunoprecipitation of the [^{35}S]methionine-labeled translation products with antialbumin was analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography.

less than 0.01 copy of albumin sequences in Novikoff hepatoma polysomal poly(A)-containing RNA, whereas in rat liver about 17,000 copies are present per cell. To corroborate these findings, as well as to determine whether the lack of albumin mRNA in Novikoff hepatoma is the result of alterations in the normal transport or a reflection of the repression of the albumin gene in these cells, a single-stranded albumin-specific DNA probe was prepared from the cloned sequence as described in Materials and Methods. Figure 4 shows the titration of a fixed amount of ^{32}P-labeled, single-stranded albumin cDNA with increasing amounts of rat liver and Novikoff hepatoma polyadenylated mRNA and total RNA. Both mRNA and total Novikoff hepatoma RNA showed no hybridization even at an RNA/cDNA ratio of 2×10^5. When the RNA/cDNA ratio was increased to about 8×10^5, hybridizations of 18 to 20% for mRNA and 7 to 9% for total RNA were observed. The titration curves represent the values obtained after subtraction of the percentage of hybridization reached using tRNA as control. This hybridization was about 10 to 12% almost throughout the reaction, except for the very high tRNA/cDNA ratios, where it reached 17% and was due to self-annealing of the cDNA probe. The titration curve with liver mRNA reached 50% hybridization at an RNA/cDNA ratio of about 72, which is about 2.4 times higher than that obtained using cDNA reverse transcribed from albumin mRNA (data not shown). Taking into account that the cloned cDNA probe (800 nucleotides) used was about 1.87 times smaller than the cDNA (1,500 nucleotides), it would be expected, theoretically, to get 50% hybridization at an RNA/cDNA ratio of about 56. This deviation from the expected value is probably the result of the effect of the cDNA length on the RNA/cDNA hybridization rate. From the titration curve of the total liver RNA, where 50% hybridization was achieved at an RNA/cDNA ratio of 5,240, and that of liver mRNA (consisting of 7% albumin sequences), it is revealed that albumin-specific sequences consist of 0.1% of the total liver RNA.

Detection of albumin sequences in RNAs of rat liver and Novikoff hepatoma blotted on nitrocellulose filter. Another possibility to confirm the repression of albumin gene in Novikoff hepatoma is the hybridization of RNA blotted on nitrocellulose paper with nick-translated albumin insert. Figure 5 shows the autoradiogram obtained from such filter hybridization of normal rat liver and Novikoff hepatoma polysomal poly(A)-containing RNA and nuclear RNA with ^{32}P-albumin cloned probe. The amount of Novikoff RNA used was 40 μg, which is 2×10^5-fold more than the smallest amount of liver RNA used. As it is clearly shown, no albumin band could be detected in Novikoff hepatoma. The band shown in Novikoff below the relevant position for albumin in normal liver corresponds to 18S and probably represents the background caused by mismatching with the banded 18S

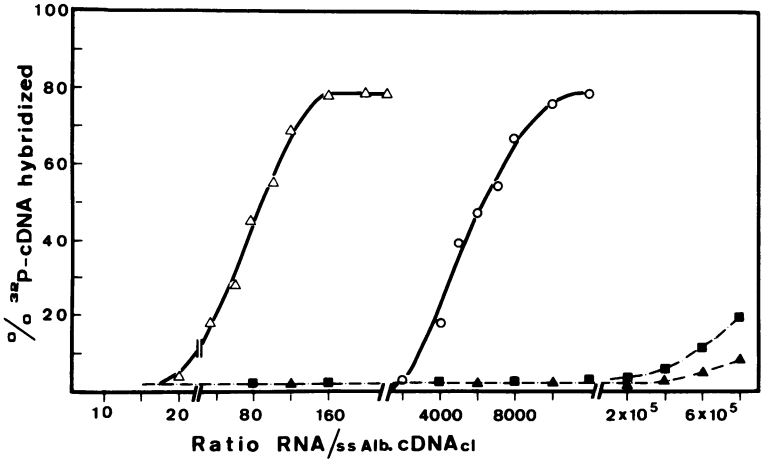

FIG. 4. Titration of single-stranded, cloned in pBR322, albumin cDNA with rat liver and Novikoff hepatoma polyadenylated mRNA and total cellular RNA. The double-stranded albumin cDNA cloned in pBR322 was excised by *PstI* digestion of the plasmid, and single-stranded probe (^{32}P-ss-albumin cDNA) was prepared as described in the text. A fixed amount of ^{32}P-ss-albumin cDNA (3,000 cpm corresponding to ~40 pg) was hybridized with increasing amounts of RNA, and the extent of hybridization was assayed by S1 nuclease digestion. Symbols: △, rat liver polyadenylated mRNA; ○, rat liver total RNA; ■, Novikoff hepatoma polyadenylated mRNA; ▲, Novikoff hepatoma total RNA.

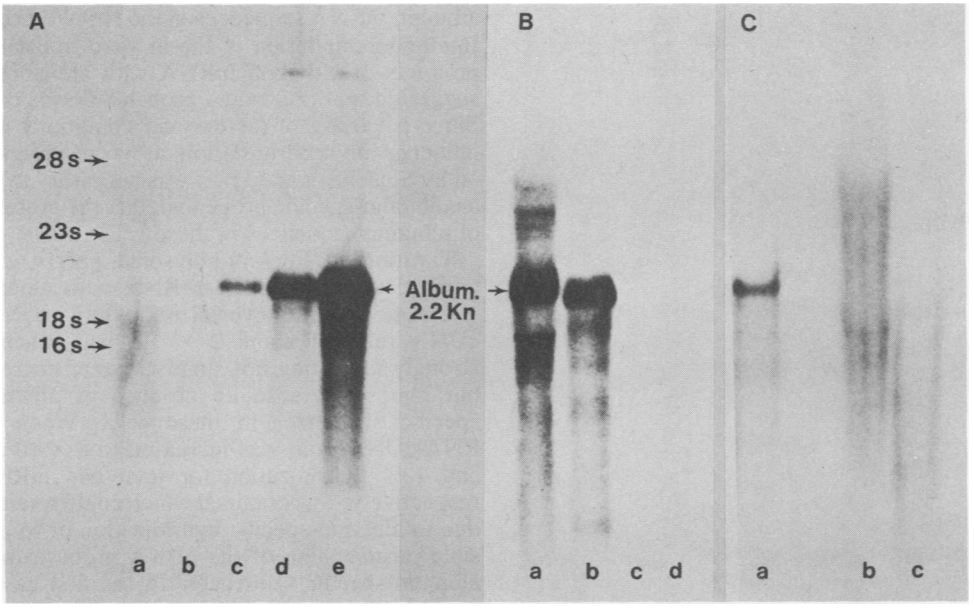

FIG. 5. RNA blotting on nitrocellulose paper for detection of albumin sequences in polysomal and nuclear RNA populations. RNAs, separated in 1.3% agarose-2.2 M formaldehyde gels, were transferred to nitrocellulose paper, hybridized with [^{32}P]albumin insert cloned in pBR322 plasmid, and autoradiographed. A. (a) 40 μg of polyadenylated Novikoff mRNA; (b) 200 pg of polyadenylated liver mRNA; (c) 2 ng of polyadenylated liver mRNA; (d) 20 ng of polyadenylated liver mRNA; (e) 200 ng of polyadenylated liver mRNA. B. (a) 4 μg of liver nuclear RNA; (b) 0.1 μg of polyadenylated liver mRNA; (c) 30 μg of Novikoff nuclear RNA; (d) 20 μg of polyadenylated Novikoff nuclear RNA. C. (a) 20 ng of polyadenylated liver mRNA; (b) 50 μg of total Novikoff RNA; (c) 30 μg of total polyadenylated Novikoff RNA.

rRNA. The overall background observed in the case of Novikoff was expected since the amount of RNA used was very high.

No albumin-specific sequence can be detected either in the nucleus or in the total RNA of Novikoff hepatoma cells. Assuming no rapid turnover, specific for albumin, all of the above results in combination with the titrations by liquid hybridization as well as the immunoprecipitation experiments argue strongly for repression of the albumin gene in Novikoff hepatoma cells.

In the case of normal liver mRNA blots (Fig. 5), several albumin-specific bands of higher molecular weight than the mature mRNA could be demonstrated (the most prominent having a length of 3,900 nucleotides), probably corresponding to precursor albumin sequences and the different splicing products. These findings, however, are only preliminary and need more investigation.

Normal rat liver and Novikoff hepatoma genome blots. The studies above provided strong evidence for repression of the albumin gene in the Novikoff hepatoma cells. To further examine if this repression is the result of any possible deletion or rearrangement in the albumin gene in these cells, genomic DNA blots were carried out.

High-molecular-weight nuclear DNA from rat liver and Novikoff hepatoma were digested with *Eco*RI and *Hin*dIII restriction endonucleases, and the fragments were separated by electrophoresis on agarose gels and blotted on nitrocellulose filters, which were then hybridized to cloned albumin cDNA labeled with ^{32}P by nick translation. No differences were observed between the hybridization patterns obtained with rat liver and Novikoff hepatoma DNA (Fig. 6). Hybridization of the albumin probe with the *Eco*RI-restricted DNA fragments revealed two bands with sizes of 4 and 1.6 kilobases (kb), as was expected from the work of Sargent et al. (28), who obtained the same bands after hybridizing the albumin genome clone with the 3′ fragment of the albumin cDNA clone. The only difference observed in the *Eco*RI pattern between rat liver and Novikoff hepatoma albumin gene was that the 4-kb fragment band is less intense in Novikoff hepatoma than in rat liver. This reflects no alteration in the albumin gene structure as it will be discussed further in the

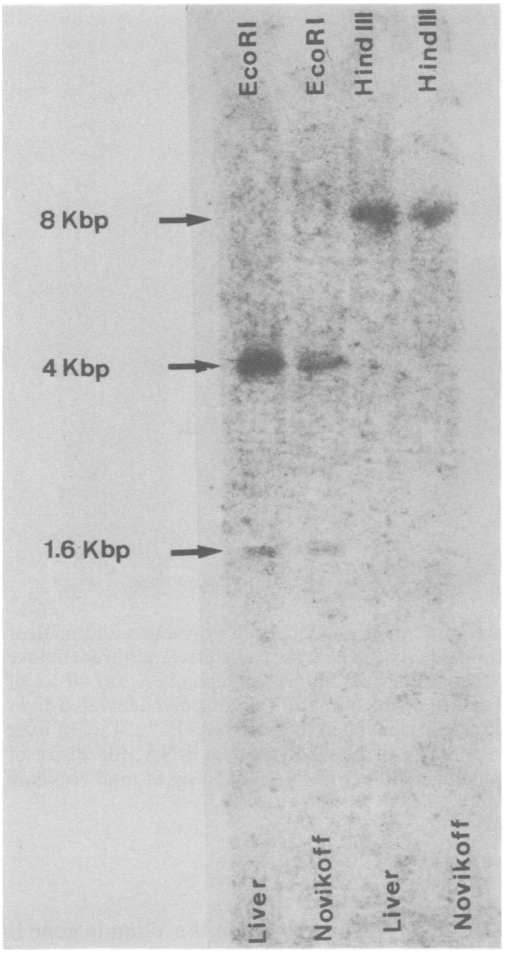

FIG. 6. Southern blots of rat liver and Novikoff hepatoma genomic DNA using cloned albumin cDNA probe. The rat liver and Novikoff hepatoma DNAs, digested with *Eco*RI and *Hin*dIII endonucleases and separated on 0.8% agarose gel, were transferred to nitrocellulose sheets. Nick-translated albumin cDNA probe was hybridized to the DNA on the nitrocellulose sheets, which were then autoradiographed.

following section. The *Hin*dIII pattern, which shows only one band of 8-kb size, is identical in both genomes.

DISCUSSION

Albumin mRNA belongs to the highest abundance class of liver mRNA sequences (11, 36). As previously published (5, 12), this class is lost or extensively decreased in frequency in Novikoff hepatoma cells. That was the first indication that the loss of the capacity of Novikoff hepatoma cells to synthesize albumin most probably is not the result of translational control but could be associated with a concomitant absence of albumin mRNA sequences in the Novikoff cells. Immunoprecipitation of the in vitro translation products of Novikoff mRNA with antialbumin suggested that this would probably be the case. Since a variety of factors and conditions may influence in vitro translation, as has been reported by Shafritz et al. (32), it was necessary to use an albumin-specific probe to detect the presence of albumin sequences in these cells.

Titration of Novikoff polysomal poly(A)-containing RNA and nuclear RNA with albumin cDNA revealed no hybridization until an RNA/cDNA ratio of about 2×10^5 was reached, strongly indicating not simply a great decrease but almost an absolute absence of albumin-specific sequences in these cells. When the RNA/cDNA ratio was increased to 8×10^5, 10 and 20% hybridization for total and mRNA, respectively, was obtained which could be either due to albumin-specific hybridization or to possible mismatching of the cDNA probe to non-albumin-specific sequences. In the first case it could be calculated that less than 0.01 copy of albumin sequences per cell is present in Novikoff hepatoma polysomes, whereas in normal rat liver about 17,000 exist. The titrations with liver mRNA revealed that about 7% of the total liver polysomal poly(A)-containing RNA consists of albumin polyadenylated mRNA. This finding is in agreement with that obtained by Hofer et al. (11), Sala-Trepat et al. (26), and Tse et al. (36), whose values were between 7 and 10%.

Using this cloned albumin cDNA, the titration data were verified by another approach according to which detection of specific RNA species was accomplished by transferring electrophoretically separated RNA species to a solid phase, similar to the Southern technique, and hybridizing them with radioactive complementary DNA. Using this method, it was found that even when a 2×10^5 times greater amount of Novikoff mRNA than the lowest detectable amount of liver mRNA was analyzed, no albumin band could be found in the autoradiogram obtained even after prolonged exposure. The high background shown in the case of Novikoff hepatoma mRNA (Fig. 5), particularly with regard to 18S rRNA, suggested that part of the hybridization obtained by Novikoff mRNA at very high RNA/cDNA ratios in the titration experiment might be due to mismatching. All of the above results taken together suggest that the loss of the capacity of Novikoff hepatoma cells to synthesize albumin is consistent with the absence of the corresponding functional mRNAs in these cells, and that translation control does not appear to be a factor in the control of gene expression of albumin. These observations are in agreement with the findings reported from other laboratories using Morris hepatoma 7777, which has

not totally lost the capacity to synthesize albumin but is, however, characterized by an extensive decrease in its expression which is mainly correlated with the steady-state concentration of the albumin mRNA (36).

However, the possibility that albumin sequences are transcribed but rapidly turned over cannot be excluded, despite the fact that other authors have recently reported that they had not found any evidence of selective degradation of albumin transcripts in nonpermissive states (30).

Titrations of albumin cDNA with liver total and poly(A)-containing RNA revealed that about 60% of the steady-state nuclear albumin-specific sequences lack poly(A). Possibly part of this RNA corresponds to the high non-polyadenylated fraction (about 40%) of the albumin mRNA found by Tse et al. (36) and Sala-Trepat et al. (26), thus suggesting that perhaps the deadenylated albumin mRNA molecules reflect the lack of poly(A) addition to some of the initial mRNA transcripts (17) and not aging of the mRNA molecules (33).

To further determine whether the observed repression of the albumin gene in the Novikoff hepatoma cells is the result of any deletion or rearrangement in the albumin gene, cloned albumin cDNA probe was hybridized to *Eco*RI and *Hin*dIII digested, filter-bound rat and Novikoff hepatoma DNA. No appreciable differences were detected in the hybridization patterns between the two cell types. The possibility that the less intense band of the 4-kb *Eco*RI fragment observed in Novikoff hepatoma reflects a possible structural alteration in the albumin gene is excluded, since this fragment belongs to the greater *Hin*dIII fragment (8 kb long), as revealed from the known albumin restriction site map (28), which is identical in the two cells. The patterns we obtained using rat liver DNA are in very good agreement with those obtained by Sargent et al. (28), thus confirming once more that the present albumin clone corresponds to the 3′ end. The similarity in the hybridization patterns displayed by DNA from normal liver and hepatoma cells suggested that the gross organization of the albumin gene is not permanently altered by processes involved in the shut-off of albumin RNA synthesis in these cells. It should be mentioned, however, that the present genomic blots cannot exclude small size alterations in the albumin gene of Novikoff hepatoma, for example those smaller than 100 base pairs. For such studies isolation of the albumin gene from both cells and restriction site mapping as well as sequencing would be required. Furthermore, although a great part of the albumin gene (8 kb) seems identical in both genomes, alterations in the other portion of the gene (5′ region) cannot be excluded using only the present available probe. Nevertheless, since similar results have been reported with Morris hepatoma (28), it seems likely that permanent deletions, insertions, or rearrangements in the albumin gene are not responsible for the changes in the normal phenotypic expression of albumin gene during liver carcinogenesis.

As suggested above, it is highly likely that permanent alterations in the gross organization of the albumin gene are not responsible for the inability of the RNA polymerase to synthesize albumin RNA. Therefore, there might be other factors which do not allow RNA polymerase to transcribe preferentially the albumin gene in these hepatoma cells. It has been shown that "active" nonribosomal genes have a chromatin conformation which renders them sensitive to digestion by DNase I (16) and preferential cleavage by micrococcal nuclease (2). Thus, for instance, globin genes can be digested in nuclei of chicken erythrocytes (38) and mouse erythroleukemia and fetal liver cells (18), but not in nuclei of chicken fibroblasts and brain or mouse adult liver and hepatoma. Also, active and inactive ovalbumin genes (20) can be distinguished in this way. It could be possible, therefore, that the inactivation of the albumin gene in the Novikoff hepatoma cells may be due to such conformation of the albumin chromatin which does not allow RNA polymerase to work. DNase I sensitivity experiments can give an insight into the validity of such hypothesis. Possible methylation of the gene should also be examined.

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