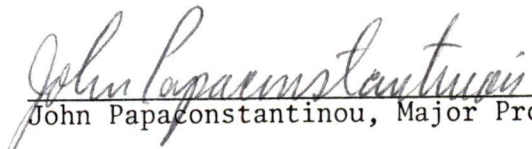
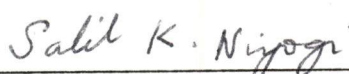


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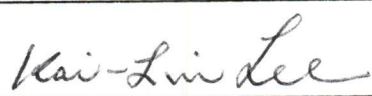
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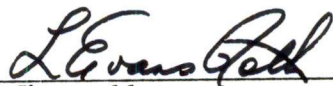
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ALBUMIN MESSENGER RNA IN MOUSE LIVER AND
A MOUSE HEPATOMA CELL LINE

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Peter C. Brown

June 1979

1979

DEDICATED TO
LOUISE HOLLOWAY BROWN

whose love and unquestioned support are constant

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ABSTRACT

The subject of this dissertation concerns the isolation and characterization of mouse liver albumin messenger RNA (mRNA), the synthesis of complementary DNA (cDNA), and the use of this cDNA in the analysis of albumin synthesis in a cell line derived from a mouse hepatoma.

Albumin mRNA was purified from mouse liver by specific immunoprecipitation of albumin synthesizing polysomes. Initial investigations, employing ^{125}I -labeled anti-mouse serum albumin, revealed that these antibodies specifically bind to albumin-synthesizing polysomes by immunologically recognizing nascent albumin polypeptides. Albumin mRNA, isolated by immunoprecipitation and selection by oligo (dT)-cellulose chromatography, was translated in a cell-free system derived from wheat germ and was shown to be enriched approximately 10-12 fold when compared to total liver polysomal RNA. This poly(A)-containing albumin mRNA exhibited a sedimentation coefficient of approximately 17S, as judged by sedimentation in aqueous sucrose gradients, and the purity of the isolated species was in excess of 80%.

Approximately full length cDNA to albumin mRNA was synthesized using avian myeloblastosis virus reverse transcriptase. This cDNA probe, when reassociated with template albumin mRNA, displayed the kinetics expected of a molecule of about 80% purity, $\text{Rot}_{1/2} = 2 \times 10^{-3} \text{ mol} \cdot \text{sec} \cdot \text{l}^{-1}$. Employing this probe in RNA excess hybridizations to mouse liver and to hepatoma cell cytoplasmic RNA, albumin mRNA levels in

liver were approximately 10-fold higher than albumin mRNA levels in hepatoma cells.

The effects of hydrocortisone and of analogs of cyclic AMP on albumin synthesis in hepatoma cells was investigated. Albumin synthesis was increased about 2.5 and 3 fold by hydrocortisone and cyclic AMP, respectively, after 72 hours of treatment with each compound. Combined administration of these drugs resulted in a 10-fold increase in albumin synthesis, an increase that was greater than additive for the two compounds given separately. Corresponding increases in albumin mRNA levels, as measured by RNA excess hybridizations to albumin cDNA, were in complete agreement with increases in albumin synthesis, suggesting that both hydrocortisone and cyclic AMP act to increase translatable albumin mRNA in hepatoma cells and therefore may be important regulators of albumin synthesis in vivo.

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CHAPTER I

INTRODUCTION

As every beginning student of biology learns, the flow of genetic information passes from DNA, to RNA and ultimately into the synthesis of protein. Just as the flow of information has been partitioned among these molecules, so has the study of the molecular biology of gene expression been divided. Undoubtedly, the most thoroughly studied area, due largely to lack of technical limitations, has been and continues to be the area of protein metabolism which includes the synthesis, modification, transport and degradation of specific proteins. Of particular interest is the influence of processes of development, aging, neoplastic transformation and a plethora of humoral factors on the natural history of enzymes and structural proteins. Protein metabolism may be considered the ultimate manifestation of a gene or a combination of genes and results in the biochemical phenotype for that system under specific conditions or at a distinct time.

A second area, and one separated spatially and temporally from protein metabolism, is messenger RNA (mRNA) metabolism. mRNA is synthesized (transcribed) in the nucleus, undergoes extensive processing and modification, and is transported to the cytoplasm where translation into protein occurs. Early investigators in this area were only capable of studying total mRNA populations due to the inability to distinguish individual species of mRNA from the total cellular complement of mRNA. With the development of mRNA-dependent-cell free translation

systems, levels of functional and specific mRNA could be determined by immunochemical quantitation of protein synthesized under the direction of added mRNA. A second approach in the study of the metabolism of specific mRNA species is by hybridization techniques with highly specific complementary DNA (cDNA) probes. These probes have become available with the developing methods of specific mRNA isolation and the discovery of viral RNA-dependent DNA polymerase (reverse transcriptase). Depending on the hybridization protocol used, specific mRNA synthesis, degradation and steady-state levels may be determined. By investigating changes in specific mRNA metabolism under conditions known to change the ultimate expression of certain genes, a new dimension in understanding is achieved.

A third area of investigation is at the level of the gene. Studies in this area have been complicated by the complexity of the eukaryotic genome, the diploid nature of the gene arrangement, and the difficulties in obtaining mutant systems. In addition, a major technical breakthrough is needed in the development of in vitro transcription systems of high fidelity and efficiency in order to study directly the actions of various proposed regulatory factors on the synthesis of specific mRNA. Advances in molecular cloning and related techniques have facilitated the isolation and characterization of naturally occurring gene sequences. These studies are providing information of the primary and secondary structure of the genes themselves and adjacent regulatory elements. In addition, physical studies are elucidating the higher order structures involved in chromatin organization and may provide important structure/function relationships in the genome of higher organisms.

Serum albumin comprises about 50% of serum protein and its synthesis in the liver is approximately 10% of the total synthetic capacity of that organ (105). The albumin molecule, because of the availability of large quantities of highly purified material, has been one of the more thoroughly studied proteins. Albumin is extremely soluble in water and is characterized by its high negative charge (10 at pH 7.4) and lack of carbohydrate (116). In humans, albumin is composed of 580 amino acids which have been completely sequenced, and the protein has a molecular weight of about 68,000 (18). Various functions have been ascribed to albumin including the maintenance of osmotic pressure and as a carrier of metallic and other ions, fatty acids, amino acids, metabolites, bilirubin, enzymes, drugs and hormones. In addition, albumin has been postulated to function as a store of readily available amino acids during periods of starvation (106).

Clinically, serum albumin has been used as a general indicator of vascular integrity, nutritional status and liver function in health and disease (106). Knowledge of the events surrounding albumin synthesis is important because of the significance of albumin to the organism and because of the relatively massive quantities of albumin synthesized. In man, albumin synthesis ranges from 120 to 300 mg per kg body weight per day (116). The albumin system has been made more attractive for study because of the relative ease with which purified albumin, highly specific antibodies to albumin and purified albumin mRNA can be obtained (141).

The synthesis of albumin begins before birth in humans and in mice (67, 116) and the regulation of albumin synthesis in adult life is the

result of complex interactions of nutritional and hormonal factors. The availability of amino acids has been shown to affect total protein and albumin synthesis and conditions of starvation and protein deficiency severely limit the synthetic activity of the liver (116). It is particularly interesting that the essential amino acid tryptophan seems to be a rate-limiting reagent in albumin and total protein synthesis. In the isolated, perfused livers from fasted animals, albumin synthesis is depressed approximately 50% compared to livers from control animals. Of all the amino acids tested, only the addition of tryptophan to the perfusate restored synthesis to control levels (115). This effect on general depression of protein synthesis is probably explained by the fact that the whole body content of tryptophan is by far the lowest of any amino acid (88). Upon refeeding fasted rats, albumin synthesis relative to total protein synthesis is immediately increased 3-fold. This differential effect on albumin synthesis is interpreted as evidence for the extreme stability of albumin mRNA, since even greater increases in albumin synthesis occur after refeeding fasted rats whose capacity for new mRNA synthesis is inhibited by Actinomycin D treatment (150).

The importance of the glucocorticoid hydrocortisone in maintaining serum albumin levels was recognized as early as 1942 in studies of hypophysectomized and adrenalectomized rats (69). Later studies in vivo showed that increased synthesis and degradation of albumin are associated with hydrocortisone treatment (24, 114). Thus, serum albumin levels are not drastically altered by hydrocortisone treatment, even though net synthesis has been increased. In some as yet undefined

manner, synthesis and degradation are coordinated to stabilize serum albumin levels under varying hormonal conditions. Studies were extended to the isolated, perfused liver where hydrocortisone was shown to mediate increases in albumin synthesis (82). Finally, albumin synthesis in a cell line derived from a rat hepatoma was increased 4-fold by the addition of hydrocortisone to the growth media (138). The effects of pituitary growth hormone on albumin synthesis are disputed. In one study, growth hormone replacement to hypophysectomized rats was effective in increasing synthesis (65). In a contrasting study, albumin synthesis in intact rats was unaffected by growth hormone administration, whereas the synthesis of fibrinogen, another hepatic secretory protein, was augmented (57). Thyroid hormone and insulin are other factors which seem to regulate albumin synthesis and increased albumin production was observed after thyroid hormone and insulin treatment of isolated, perfused livers (59, 102). In no instance have these studies been extended to determine whether increased albumin mRNA levels might explain the observed changes in albumin synthesis by the hormones mentioned.

Albumin is representative of the class of secretory proteins in general. It has been shown by many workers that, like all serum proteins, albumin is synthesized on membrane-bound polysomes (63, 79). The events leading to the deposition of polysomes on membranes have been in dispute, but it appears that the signal hypothesis, originally proposed by Blobel (11), explains the origins of membrane-bound polysomes. In this model, mRNAs which code for secretory proteins initiate translation on free, unbound ribosomes. At the initial amino

terminus of the nascent protein, an 18-20 amino acid "signal piece" mediates the attachment of the polysome complex to membranes of the endoplasmic reticulum. Nascent protein is propelled through the membrane into the cisternae of the endoplasmic reticulum and the signal piece is cleaved by specific proteases to produce, in the case of albumin, proalbumin. Proalbumin differs from serum albumin by a 6 amino acid extension on the amino terminus (118), the function of which is unknown. From the rough endoplasmic reticulum, newly synthesized albumin progresses through the smooth endoplasmic reticulum fraction and ultimately to the Golgi complex where it is packaged into secretory vesicles (104). Recent data indicate that a calcium-dependent fusion of vesicles containing proalbumin and a cathepsin B-like protease occurs, converting proalbumin to albumin and allowing for secretion via fusion with the plasma membrane (61). In such a complicated process, albumin production could be regulated at any of these steps between synthesis and secretion.

Research in eukaryotic gene expression has benefited greatly from the establishment of permanent cell lines which maintain the regulation and synthesis of proteins which are characteristic of the cell of origin in vivo. The literature now abounds with descriptions of cell systems of this type. Some of the more prominent examples are cells derived from plasmacytomas which synthesize immunoglobulins (70); erythroleukemic cells which synthesize globin (36); melanoma cells which synthesize melanin (85); pituitary tumor cells which synthesize hormones (23); and hepatomas which synthesize inducible enzymes and serum proteins (92, 137, 138, 144). The mouse hepatoma cell line derived from hepatoma

BW7756 is one of these cell lines and is designated Hepa (9). After many years in continuous culture, clones of this line continue to synthesize and secrete the serum proteins albumin (9, 98), α -fetoprotein (27), transferrin (99), and ceruloplasmin (27), in addition to other proteins not yet characterized. These cells are of particular value in studying factors which affect the synthesis of serum proteins because alpha fetoprotein is only synthesized during fetal development, after liver injury and in liver cancer (39), while the other proteins are synthesized continuously. The advantages in working with tissue culture systems include the ability to define more precisely the various chemical and physical parameters of the immediate environment of the cell, the reduction of indirect effects due to factors such as drug metabolism outside the cells under study, and the ability to investigate processes in single cells and synchronized populations of cells. Technically, tissue culture systems enable rapid and extensive labeling of newly synthesized cellular components and the ability to quickly process these samples for detailed kinetic studies.

The applicability of results obtained from tissue culture systems to in vivo situations has been and continues to be a subject of contention. With few exceptions, permanent cell lines have been derived from neoplastic tissues. Those cell lines derived from normal cells generally become transformed, allowing them to grow in culture and are tumorigenic when injected into syngeneic hosts. An aberration common to most cell lines is the development of severe chromosome abnormalities. While generalizations about tissue culture systems are dangerous and can be misleading, the applicability of the study of general biochemical pathways, the

so-called "housekeeping" functions, seems assured. Differentiated functions and "luxury proteins" not essential to the continuance of living processes must be viewed with caution. Negative evidence for a certain process or regulatory event related to differentiated function must be applied to only that system under study, since "dedifferentiation" in tissue culture seems to occur in many cases (100). Positive evidence of effects of proposed regulators of differentiated function must be rationalized in conjunction with the proliferative capacity or state of the cell. Many cell functions are growth stage and cell cycle stage specific (44, 83), and the effects of a regulator on specific function may be related to alterations in parameters of cell growth.

The sequence of chapters in this dissertation follow the order in which the work was performed and, as such, is a chronological record of progress. In Chapter II, the ability of antibodies to specifically bind to albumin synthesizing polysomes from liver and Hepa-2 cells is determined. An indication of quantitative differences in albumin synthesizing polysomes in the two cell sources is presented. In the next chapter, using specific antibody precipitation techniques, albumin synthesizing polysomes are isolated from mouse liver polysomes and albumin mRNA, extracted from these purified polysomes, is characterized with respect to purity, physical and functional properties. Chapter IV is concerned with the synthesis and characterization of albumin cDNA. In addition, nucleic acid hybridization techniques are used to compare albumin mRNA levels in liver and in Hepa-2 cells. The last chapter analyzes the effects of hydrocortisone and analogues of cyclic AMP on albumin synthesis and secretion in Hepa-2 cells and the corresponding

albumin mRNA levels determined by nucleic acid hybridization of albumin cDNA to cellular RNA. With respect to the outline of research in the molecular biology of eukaryotic gene expression in the introduction to this chapter, work described in this dissertation encompasses the first two levels of investigation; the areas of specific protein and mRNA metabolism.

CHAPTER II

IDENTIFICATION OF ALBUMIN SYNTHESIZING POLYSOMES FROM MOUSE LIVER AND A MOUSE HEPATOMA CELL LINE

I. INTRODUCTION

The successful immunoprecipitation of polysomes engaged in the synthesis of specific proteins is absolutely dependent upon the immunological recognition of nascent polysomal proteins by antibodies directed against the intact, mature protein. For example, it is conceivable that antigenic determinants of serum albumin would not be apparent in nascent albumin molecules because of the incomplete nature of the latter. In order to initially resolve this question, a series of experiments were performed to show that ^{125}I -labeled monospecific antibodies to serum albumin could specifically recognize nascent polysomal albumin in mouse liver and Hepa-2 cells.

A second reason for initiating these studies on antibody-binding to nascent albumin was an attempt to quantitate albumin synthesizing polysomes in liver and in Hepa-2 cells. From previous studies in the laboratory, it was apparent that albumin synthesis in Hepa-2 cells comprised approximately 1% of total protein synthesis (Chapter V). In rodent liver, however, albumin synthesis relative to total protein synthesis is about 8-10% (35, 105, 146, 149). It was naturally of interest to determine whether these differences in albumin synthesizing capacity were reflected in the quantity of albumin-synthesizing polysomes in liver and in Hepa-2 cells.

Although previous investigators had claimed to have bound antibody to nascent protein, their results were obscured by lack of evidence demonstrating the specificity of binding (94). Palacios et al. were the first to show specific binding of monospecific antibodies to ovalbumin synthesizing polysomes from hen oviduct (94). Subsequently, various modifications of this technique have been used to identify polysomes engaged in the synthesis of rat serum albumin (141), collagen (101), fibrinogen (12), serine dehydratase (80), and α and β globin (13). Similarly, polysomes engaged in the synthesis of fatty acid synthetase (1), and dihydrofolate reductase (3) have been identified in cultured cells. In all cases, the antibody binding has been shown to involve the specific interaction of antibody with nascent polypeptide chains.

Results from this study agree with previous findings in that monospecific antibodies to albumin can recognize nascent albumin in mouse liver polysomes and in Hepa-2 polysomes. Furthermore, differences in albumin synthesis of liver and Hepa-2 cells are reflected in the extent of antibody binding to polysomes from each source. The results of this chapter have been published in more abbreviated form (20).

II. EXPERIMENTAL PROCEDURES

Purification of Mouse Serum Albumin

All operations, unless specified, were performed at room temperature. Albumin (MSA) was extracted from mouse plasma by the method of Debro et al. (29), and further purified by modifications of the technique of Taylor and Schimke (139). Plasma was diluted 1:1

with phosphate-buffered-saline (PBS, 0.15 M NaCl, 0.01 M Na_2HPO_4 pH 7.2) and precipitated while being constantly stirred by the addition of 4 volumes of 10% trichloroacetic acid (TCA). After centrifugation ($1500 \times g$ for 10 min), the precipitate was washed with 5 volumes of 5% TCA and re-centrifuged as above. The resulting pellet was resuspended in 5 volumes of 95% ethanol containing 1% TCA. After 30 minutes of stirring, the mixture was centrifuged ($7000 g \times 10 \text{ min}$) and the supernatant removed and dialyzed overnight against water (4°C , 3 changes, 4 liters each change). After dialysis, the solution was cleared by centrifugation, neutralized by the addition of dilute base, adjusted to 0.15 M NaCl by the addition of solid NaCl, and brought to 40% saturation by the addition of neutralized saturated ammonium sulfate (AmSO_4). After 30 minutes of stirring, the suspension was centrifuged ($10,000 g \times 10 \text{ min}$) and the AmSO_4 concentration increased to 66% in the supernatant fraction. After adjusting the pH to 5.0 by the addition of dilute acetic acid, the resulting precipitate was collected by centrifugation, dissolved and dialyzed against 0.035 M TRIS, pH 7.4, overnight. The material was chromatographed on DEAE cellulose (BioRad, DEAE-C) utilizing a 0.035-0.35 M Tris-HCl gradient, pH 7.4. Column elution was monitored spectrophotometrically at 280 m μ and peak fractions eluting at about 0.20-0.25 M TRIS were pooled, dialyzed against PBS and concentrated by ultrafiltration (Amicon PM-30 membrane). This final material was chromatographed on Sephadex G-100 (Pharmacia) and the material eluting in the void volume discarded. The material eluting in the retained volume was identified as mouse serum albumin by its reaction in double diffusion gels with antibody to authentic mouse serum albumin.

Furthermore, this purified mouse serum albumin coelectrophoresed with authentic mouse albumin (m.w. 68,000) as a single homogeneous band on 10.5% SDS-polyacrylamide gels.

Preparation of Antiserum

Antiserum to purified mouse serum albumin was raised in rabbits. Albumin (approximately 3.5 mg) was emulsified with an equal volume of Freund's complete adjuvant and injected at several flank locations subcutaneously. Injections were repeated at 2 week intervals with Freund's incomplete adjuvant. After 4 injections and at 2 week intervals thereafter, rabbits were bled from the ear and blood allowed to coagulate overnight in the cold. Serum was then collected after centrifugation ($2000\text{ g} \times 10\text{ min}$). All subsequent operations were performed at 4°C .

The IgG fraction was prepared from the serum by AmSO_4 precipitation and by DEAE cellulose chromatography (94). Serum was diluted 1:1 with PBS and adjusted to 40% saturation with neutralized, saturated AmSO_4 . The precipitate was stirred 60 min, collected by centrifugation and washed with an equal volume of 40% saturated AmSO_4 . The final precipitate was dissolved in a volume of buffer C (10 mM TRIS, pH 7.2, 15 mM NaCl) equal to one-half the serum volume and dialyzed against the same buffer overnight. This crude IgG fraction was then passed through a column ($2.5 \times 20\text{ cm}$) of DEAE-cellulose equilibrated and eluted in buffer C. The breakthrough material representing purified IgG was then adjusted to 0.15 M NaCl and saved for further purification.

Affinity Chromatography of Albumin Antibodies

Purified albumin was covalently coupled to Sepharose 4B (Pharmacia) by the method of Cuatrecasas (26) and used as an insoluble antigen matrix to isolate specific albumin antibodies from the IgG fraction of immune serum. Activated Sepharose was prepared by mixing powdered cyanogen bromide (CNBr, Eastman Chemicals) in the proportions of 200 mg CNBr per packed milliliter of Sepharose suspended in water. The pH was maintained at 11.0 by the addition of 2 M NaOH and the temperature never allowed to exceed 25°C. Upon dissolution of the CNBr and the cessation of the need to maintain pH control (approximately 10 min), the activated Sepharose was washed with 10-15 volumes of ice-cold coupling buffer (0.1 M sodium borate, pH 9.0) and mixed with purified albumin previously equilibrated in coupling buffer (1-5 mg albumin per milliliter of Sepharose) and allowed to gently stir in the cold overnight. The preparation was sequentially washed (2-3 volumes per wash) with buffer 8 (0.1 M sodium borate, pH 8.0, 1.0 M NaCl), buffer 4 (0.1 M sodium acetate, pH 4.0, 1.0 M NaCl), elution buffer (0.1 M glycine-HCl, pH 2.8) and finally PBS. Routinely, 20-25% of the albumin failed to couple to the Sepharose and was detected in the initial washes. The final preparation was mixed with immune IgG and allowed to stir gently at room temperature for 1 hour. The mixture was then poured into a small column and elution monitored spectrophotometrically. The column was washed with PBS, buffer 8, buffer 4, PBS and elution buffer and each wash was continued until baseline readings of eluted materials were achieved. The antibody eluted in the elution buffer was quickly neutralized, dialyzed against buffer C and concentrated by

ultrafiltration. Approximately 20% of the applied IgG fraction was recovered in the elution buffer.

Purified antibody was rendered ribonuclease-free by passage of this material through a column (1 × 5 cm) of DEAE-cellulose overlaid with an equal volume of carboxymethyl-cellulose (Whatman CM-23) both equilibrated and autoclaved in buffer C (90). The final preparation was stored in small aliquots at -20°C.

Iodination of Albumin Antibodies

Iodination of purified anti-albumin was essentially as described by Palacios et al. (94), with minor modifications. All antibodies were adjusted to 0.05 M Na_2HPO_4 pH 7.5, by dialysis. Reaction mixtures (2 ml) contained 5-10 mg antibody, 0.08 mg lactoperoxidase (Sigma Chemicals), 2 mCi K^{125}I (Amersham-Searle), 10 μM H_2O_2 (freshly prepared), and 0.05 M Na_2HPO_4 , pH 7.5. After 30 minutes at room temperature, the reaction mixture was chromatographed on Sephadex G-100 (1.4 × 40 cm column) eluted in buffer C and the breakthrough material concentrated by AmSO_4 precipitation at 50% saturation. The precipitate was taken up and dialyzed against buffer C and rendered ribonuclease-free as before. The final preparation was dialyzed, under RNase-free conditions, against polysome buffer 100 (PB_{100} , 0.05 M Tris, pH 7.1, 0.025 M NaCl, 0.005 M MgCl_2 and 100 $\mu\text{g/ml}$ sodium heparin) and stored at 4°C until used.

Preparation of Polysomes

To reduce RNase contamination, all labware coming in contact with susceptible preparations was treated with a freshly prepared solution of 0.1% diethylpyrocarbonate (DEPC, Sigma Chemicals) and dry heat treatment

at 170°C for a minimum of 5 hours. All solutions, if possible, were autoclaved prior to use. Disposable plasticware was used wherever suitable.

Liver and brain polysomes were prepared from young adult mice by a modification of the technique of Taylor and Schimke (135). Tissue was homogenized in 0.25 M sucrose in PB₁₀₀ employing a motor-driven Potter-Elvehjem homogenizer. A post-mitochondrial supernatant was prepared by centrifugation of the homogenate ($14,500 \times g$ for 15 min). The supernatant was adjusted to 1% Triton X-100 and sodium deoxycholate and gently mixed by re-pipetting (86). After 10 minutes at 4°C, this mixture (6 ml) was layered over a discontinuous sucrose gradient consisting of 2.5 M sucrose (2 ml), 1.0 M sucrose (5 ml), and 0.5 M sucrose (0.5 ml), all in PB₁₀₀. After centrifugation (SW-40 rotor, 40,000 rpm for 90 min) polysomes were withdrawn at the 2.5 M sucrose interface and dialyzed at 0°C for 12 hours against PB₁₀₀.

Hepa-2 cells were grown to confluency (approximately 15×10^6 cells per 100 mm Petri dish) in Waymouth's MAB 87/3 medium supplemented with 10% fetal calf serum (Grand Island Biological Co.) in a humidified atmosphere containing 95% air and 5% CO₂. The cells were gently scraped using a rubber policeman in 0.5 ml PB₁₀₀ and the cell suspension adjusted to 0.5% Triton X-100, gently re-pipetted, and allowed to stand on ice for 10 minutes. No intact cells were visible after this lysis and harvesting and lysis, and nuclei appeared to be intact. A post-mitochondrial supernatant was prepared and polysomes isolated as above. Approximately 2-3 A₂₆₀ units of polysomes could be recovered from each dish and the A₂₆₀/A₂₈₀ ratio of these polysomes routinely exceeded 1.75. From liver

tissue, 30-40 A_{260} units could be obtained from each gram of tissue and the A_{260}/A_{280} ratio was always greater than 1.70.

Binding of Antibody to Albumin Polysomes

Labeled antibodies and dialyzed polysomes were mixed and incubated for 1 hour at 0°C in a final volume of 0.75 ml. Continuous sucrose gradients were prepared (11.5 ml, 0.5-1.5 M sucrose in PB with 40 µg heparin/ml) over which was layered 1.0 ml of 0.5 M sucrose containing 0.5% Triton X-100 and sodium deoxycholate. This detergent-containing layer was helpful in reducing contamination by unbound, labeled antibody in the upper regions of the underlying gradient. The antibody-polysome mixture was layered over the detergent-containing layer and centrifuged (SW-40 rotor, 40,000 rpm for 95 minutes at 2°C). Following centrifugation, the top two layers were removed by aspiration. The gradient was displaced with 60% sucrose and monitored at 254 mµ with an ISCO absorbance monitor. Fractions (0.6 ml) were collected directly into glass scintillation vials to which Aquasol (New England Nuclear), diluted and acidified as suggested by the manufacturer, was added. Radioactivity was determined by liquid scintillation spectrometry.

III. RESULTS

Binding of ^{125}I -Labeled Antibody to Hepa-2 and Liver Polysomes

Polysomes from Hepa-2 cells were incubated with ^{125}I -labeled antibodies to albumin and the mixture centrifuged in a 0.5-1.5 M sucrose density gradient. The sedimentation profile of the total polysome preparation and the binding of various amounts of labeled

antibody are shown in Figure II-1 (A). A similar experiment with mouse liver polysomes is shown in Figure II-1 (B). In each of these cases, the peak of bound radioactivity occurs in fractions 10-12, which correspond to polysomes composed of about 9-14 ribosomes. These observations are essentially identical to the reported size of rat liver albumin polysomes identified by this technique (140).

Specificity of Antibody Binding

To demonstrate the specificity of binding of antibody to albumin synthesizing polysomes from liver and Hepa-2, a series of competition binding assays were performed and these results are displayed in Figure II-2 (A) (Hepa-2) and (B) (liver). It can be seen in these panels that the binding of labeled antibody to albumin synthesizing polysomes is effectively eliminated if the polysome preparation is preincubated with saturating amounts of unlabeled albumin antibody. In contrast, preincubation with IgG fraction from nonimmune rabbit serum causes no reduction in subsequent binding of labeled albumin antibodies from that of control polysomes with no prior incubation.

Additional evidence for the specificity of antibody binding to albumin polysomes is the inability of labeled albumin antibodies to bind to polysomes from brain, a tissue which does not synthesize albumin (Figure II-2 (C)). Finally, the data in Figure II-2 (D) shows that ribonuclease treatment, after incubation with antibody, reduces the polysomes to monomers and dimers and restricts bound radioactivity to the upper region of the gradient. These data indicate that the

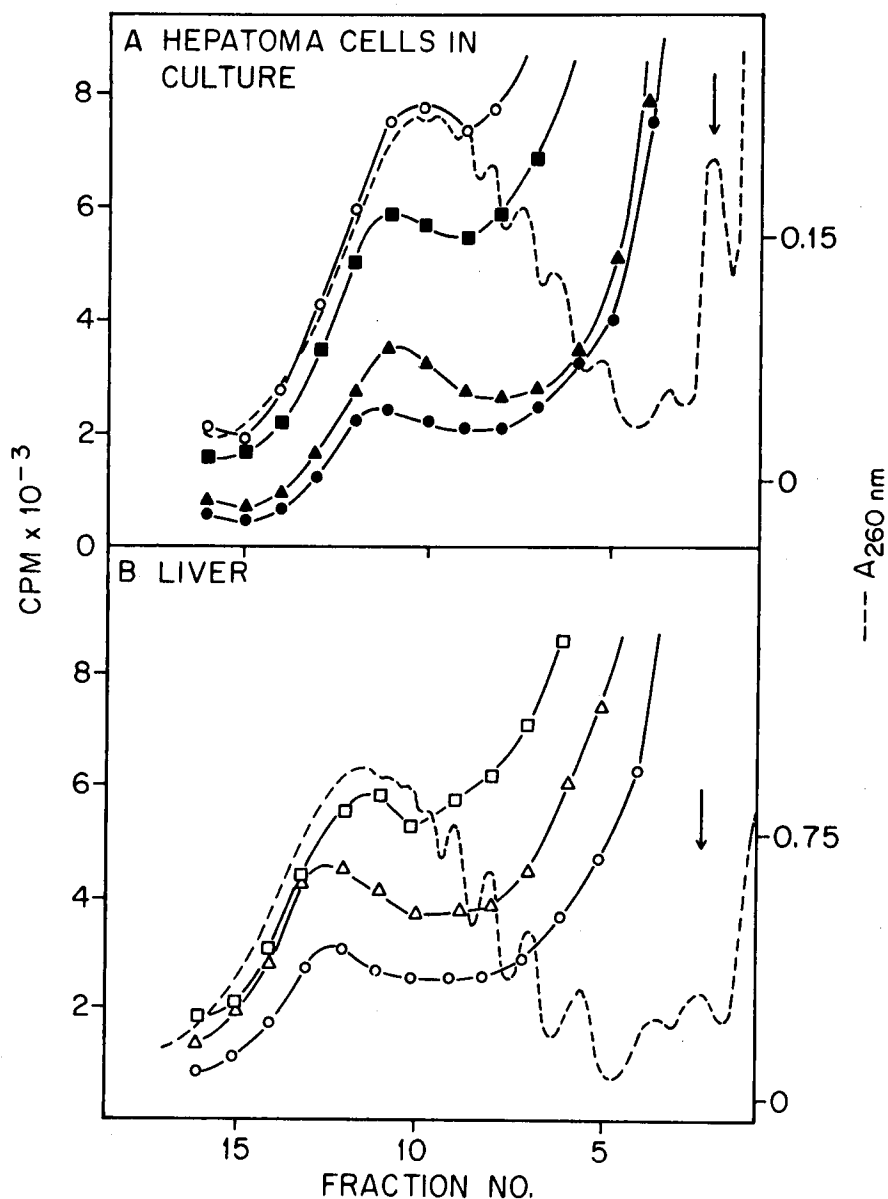


Figure II-1. Binding of ^{125}I -labeled anti-MSA to cultured mouse hepatoma cell and mouse liver polysomes.

(A) Sedimentation profile of hepatoma cell polysomes ($2.9 A_{260}$ units) incubated with ^{125}I -labeled anti-MSA (1.94×10^5 cpm/ μg). UV-absorbing material at the top of the gradient is residual detergent. ●—● 3.2 μg ; ▲—▲ 8 μg ; ■—■ 16 μg ; ○—○ 32 μg anti-MSA. Arrow indicates position of 80 S ribosomes. (B) Sedimentation profile of liver polysomes ($12.66 A_{260}$ units) incubated with ^{125}I -labeled anti-MSA (2.94×10^4 cpm/ μg). ○—○ 54 μg ; ▲—▲ 108 μg ; □—□ 216 μg .

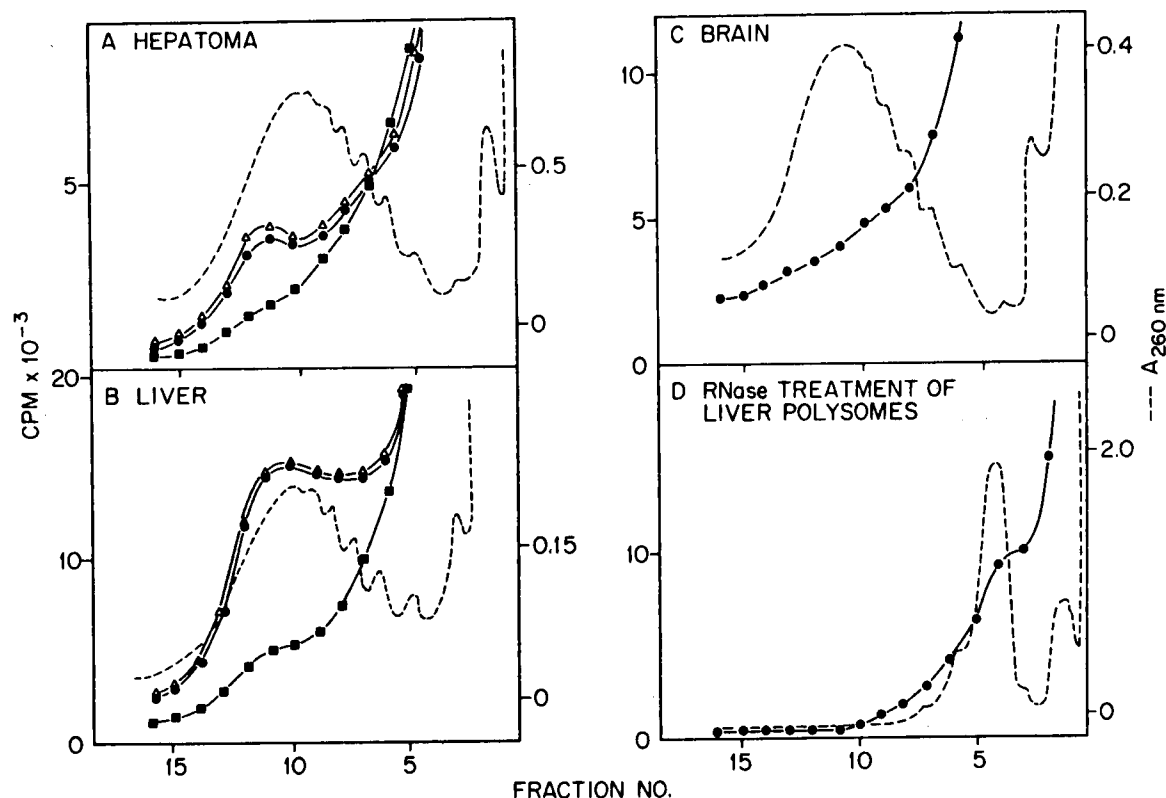


Figure II-2. Specificity of ^{125}I -labeled anti-MSA binding to polysomes.

(A) Competitive antibody binding assay. Hepatoma cell polysomes (2.6 A_{260} units) were preincubated for 45 min with nonimmunized rabbit IgG (15 μg , Δ — Δ), with unlabeled anti-MSA (15 μg , $\blacksquare$ — $\blacksquare$), or alone with no added protein ($\bullet$ — $\bullet$). ^{125}I -labeled anti-MSA (8 μg , 1.94×10^5 cpm/ μg) was added to each, incubated an additional hour, and centrifuged. (B) Competitive antibody binding assay. Liver polysomes (2.88 A_{260} units) were preincubated as above with nonimmunized rabbit IgG (54 μg , Δ — Δ), with unlabeled anti-MSA (59 μg , $\blacksquare$ — $\blacksquare$), or alone ($\bullet$ — $\bullet$). ^{125}I -labeled anti-MSA (32 μg , 1.94×10^5 cpm/ μg) was added to each and treated as above. (C) Brain polysomes (2.8 A_{260} units) were incubated with ^{125}I -labeled anti-MSA (32 μg , 1.94×10^5 cpm/ μg) and centrifuged. (D) Liver polysomes (6.3 A_{260} units) were incubated with ^{125}I -anti-MSA (54 μg , 1.94×10^5 cpm/ μg) for 1 hour and then with ribonuclease A (8 μg , Worthington Biochemicals) for 30 minutes and centrifuged.

sedimentation of intact albumin synthesizing polysomes is necessary in order to observe the radioactivity patterns seen in Figure II-1.

Comparison of Albumin Antibody Binding Capacities of Hepa-2 and Liver Polysomes

A direct comparison of the extent of antibody binding to Hepa-2 and liver polysomes is complicated by the presence of contaminating or nonspecifically bound antibody seen in the upper regions (fractions 3-7) of the sucrose gradients. That this labeled antibody is nonspecifically bound may be seen in the competitive binding assay (Figure II-2 (A), (B)), since the presence of labeled antibody is not preempted by prior incubation with unlabeled albumin antibody.

In Figure II-3 are presented the binding profiles of albumin antibody to Hepa-2 and to liver polysomes corrected for nonspecific binding. Albumin antibody was reacted with Hepa-2, liver and brain polysomes under identical conditions of antibody excess and centrifuged. Radioactivity associated with each fraction of the brain polysome gradient was subtracted from equivalent fractions of Hepa-2 and liver polysome gradients. The differences in radioactivity in each fraction provides an estimate of the specific binding of antibody to polysomes in that fraction. Thus, under identical conditions, liver polysomes appear to bind 3.4 fold more albumin antibody than Hepa-2 polysomes (5.52×10^4 total cpm for liver versus 1.61×10^4 cpm for Hepa-2). It should be pointed out that saturating amounts of antibody were used in this comparison. Examination of the competition binding assays (Figure II-2 (A), (B)) reveal that approximately 5 μ g of albumin antibody per A_{260} unit of either Hepa-2 or liver polysomes effectively saturates

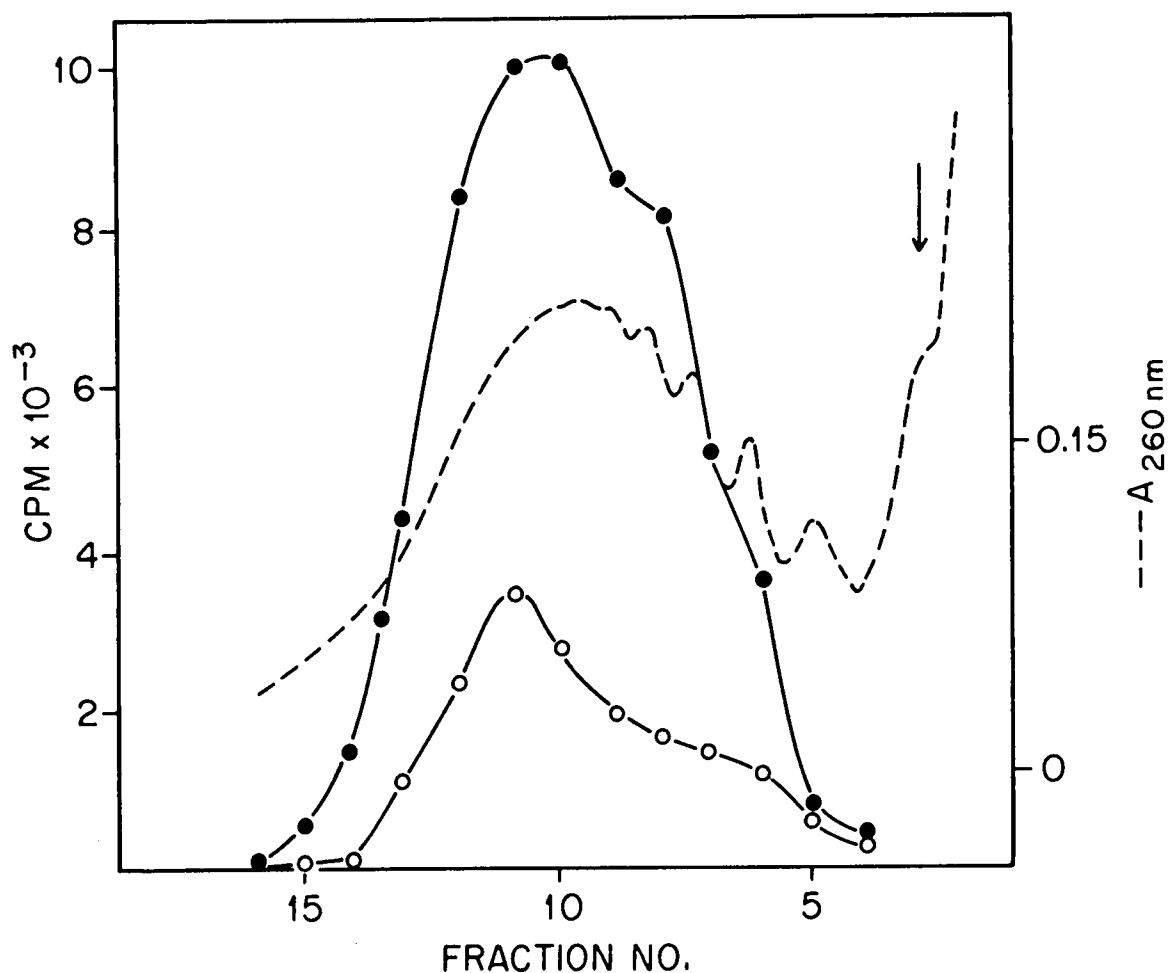


Figure II-3. Comparison of ^{125}I -labeled anti-MSA binding to hepatoma cell and liver polysomes.

^{125}I -labeled radioactivity in fractions from Figure II-2 (C) (brain polysomes, 2.8 A_{260} , 32 μg ^{125}I -labeled anti-MSA) was subtracted from equivalent fractions from Figure II-1 (A), open circles (hepatoma polysomes, 2.9 A_{260} units, 32 μg ^{125}I -labeled anti-MSA) and Figure II-2 (B), solid circles (liver polysomes 2.88 A_{260} units, 32 μg ^{125}I -labeled anti-MSA). The resulting radioactivity in each fraction represents anti-MSA binding corrected for nonspecific adsorption. Total corrected counts in the liver polysome preparation (●—●) and the hepatoma polysome (○—○) are 5.52×10^4 and 1.61×10^4 cpm, respectively. A representative polysome profile is also included for reference.

specific binding sites (nascent albumin) and prevents subsequent binding by labeled antibody. In the comparative study over twice that amount of labeled antibody was employed ($11 \mu\text{g}/A_{260}$) ensuring binding to all available sites.

IV. DISCUSSION

The above studies demonstrate that antibodies to mouse serum albumin can be used to identify albumin synthesizing polysomes from Hepa-2 cells and mouse liver. Maximal antibody binding occurred in the 9-14 ribosome region of the polysome profile from both sources. This result implies that the apparently lower level of albumin synthesis in Hepa-2 cells is not a result of reduced ribosome loading of albumin mRNA in Hepa-2 cells. Control experiments with polysomes from mouse brain and ribonuclease digestion of liver polysomes indicate that intact, albumin synthesizing polysomes are necessary in order to produce the binding patterns observed. The specificity of the antibody binding reaction in the peak region of the gradient was also verified by data from the competitive binding experiments. In summary, these experiments suggest the feasibility of employing immunological techniques in the isolation of albumin synthesizing polysomes and the eventual purification of albumin mRNA.

The comparison of albumin synthesizing polysomes in Hepa-2 cells and in liver showed that, per A_{260} unit of total polysome, albumin synthesizing polysomes were enriched 3.4 fold in liver. Based on recoveries from the polysome isolation procedures, liver cells contain approximately 1.5 fold more total polysomes than Hepa-2 cells. This calculation is based on the conversion factor 1.7×10^8 cells per gram

of liver (9). In approximate agreement is that total extractable RNA from liver cells is about 1.5-2 fold more abundant than that of Hepa-2 cells (Chapter IV). Accordingly, on a per cell basis, albumin-synthesizing polysomes are 5-6 fold more abundant in liver than in Hepa-2 cells.

In a later chapter, it is shown that albumin synthesis comprises about 1-2% of total protein synthesis in Hepa-2 cells. In liver, albumin synthesis is about 8-10% of total protein synthesis. Thus the differences in antibody binding in liver and in Hepa-2 polysomes (5-6 fold) are very close to the differences in relative albumin synthesis in the two cell types. These results would seem to preclude aberrant protein processing or altered degradation of newly synthesized albumin as an explanation for reduced albumin production in Hepa-2 cells compared to albumin synthesis in liver.

CHAPTER III

THE PURIFICATION OF MOUSE ALBUMIN MESSENGER RNA

I. INTRODUCTION

With the development of immunochemical techniques, the purification of specific eukaryotic mRNA species of undistinguished size, composition and low abundance has become feasible. Without the selectivity provided by precipitation of polysomes engaged in the synthesis of specific proteins, one is left with nonspecific physical and chemical methods for the purification of specific mRNA. Relatively few highly specialized systems are amenable to this method of specific mRNA isolation. A partial list of those mRNAs isolated by purely physical and chemical techniques includes mRNAs coding for the synthesis of globin (112), ovalbumin (111), vitellogenin (120), protamine (37), silk fibroin (136), histone (42), and alpha-crystallin (25).

The vast majority of eukaryotic mRNAs are of low abundance and are physically and chemically indistinguishable. A number of these mRNAs have been isolated by immunoprecipitation techniques in purities approaching 100%. Some of these include mRNAs coding for the synthesis of ovalbumin (95, 127), conalbumin (71), rat albumin (141), casein (48), rat α -fetoprotein (53), and α 2U-globulin (68). Partial purification of dihydrofolate reductase mRNA has also been reported (3).

The binding of albumin antibody to albumin-synthesizing polysomes was described in the previous chapter. In the present chapter, this

technique is extended and albumin-synthesizing polysomes are isolated from total mouse liver polysomes by direct immunoprecipitation. RNA was extracted from immunoprecipitated polysomes and albumin mRNA isolated by two cycles of chromatography on oligo (dT)-cellulose. This albumin mRNA was characterized by sedimentation in SDS-containing sucrose gradients and translated in a messenger-dependent cell-free system derived from commercial wheat germ. Mouse albumin mRNA sediments as a peak of material at about 17S and appears to exceed 80% purity.

II. EXPERIMENTAL PROCEDURES

Extinction coefficients used are: (1) $20 A_{260}/\text{mg RNA/ml}$; (2) mouse albumin ($0.69 A_{280}/\text{mg/ml}$); (3) rabbit and goat immunoglobulin ($1.4 A_{280}/\text{mg/ml}$). Procedures for minimizing RNase contamination of labware and solutions has been described in Chapter II.

Immunoprecipitation of Albumin Synthesizing Polysomes

The preparation of polysomes, antigen (albumin), and antialbumin IgG was done as previously described in Chapter II. Ribonuclease contamination of purified albumin and antibody was not apparent in sample polysomes exposed to each, as judged by lack of degradation in sedimentation profiles (data not shown). All operations were performed at 0-4°C. Dialyzed mouse liver polysomes ($10 A_{260}$ units/ml) were incubated for 10 minutes with nonspecific affinity-purified rabbit anti-mouse albumin IgG in buffer A (50 mM Tris, pH 7.1, at 21°C, 25 mM NaCl, 5 mM MgCl_2) with 50 $\mu\text{g/ml}$ sodium heparin (Sigma Chemical Co.), 0.4% Triton X-100 and sodium deoxycholate. Mouse serum albumin was

added, stirred gently and allowed to incubate an additional 90 min at 4°C. The resulting immunoprecipitate was collected by centrifugation at 2900 rpm, 10 min in the International centrifuge (JP-3), gently resuspended in one-fourth the original volume of buffer A with 0.2% Triton X-100, 0.1 M sucrose and sodium heparin (500 µg/ml), and recentrifuged as above.

Analytical, small-scale immunoprecipitation in a volume of 0.5 ml were performed, in duplicate, with one sample containing five A_{260} units of polysomes in addition to antibody and carrier albumin while the companion sample contained only antibody and albumin. Immunoprecipitates were dissolved in 1 ml 0.1 M NaOH and the absorbance at 260 mμ determined. Subtraction of the A_{260} reading of the immunoprecipitate without polysomes from the A_{260} reading of the immunoprecipitate with polysomes yields a net A_{260} reading of the polysomes immunoprecipitated by this technique. All precipitations were performed under conditions of antibody excess as determined independently from quantitative precipitation curves.

Preparative, large-scale immunoprecipitations were done, as described, under conditions optimized by analytical immunoprecipitation. These conditions are 360 µg of antibody and 14 µg of albumin per ml of reaction mixture. Final immunoprecipitates were resuspended and phenol-extracted.

For the preparation of total polysomal RNA, dialyzed polysomes were pelleted (type 65 rotor, 40,000 rpm for 2 hours) and phenol-extracted.

RNA Extraction, Oligo (dT)-Cellulose
Chromatography, and Centrifugation

The immunoprecipitated polysomes were solubilized in SDS-buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 100 mM NaCl, 0.5% SDS) to give a solution equivalent to 15-20 A_{260} units of polysomes/ml and extracted in phenol-chloroform by the procedure of Perry et al. (103). An equal volume of a phenol:chloroform mixture (1:1, saturated with 0.01 M sodium acetate, pH 6.0, 0.1 M NaCl, 0.001 M EDTA) was combined with solubilized polysomes and mixed for 5 min at room temperature. The emulsion was centrifuged (14,000 g $\times$ 10 min) and the aqueous phase removed. The organic layer and interface were re-extracted with one-half volume of SDS solution used to solubilize the polysomes. A second aqueous phase was prepared and pooled with the first aqueous phase. These combined aqueous phases were extracted with 1.5 volumes of phenol:chloroform, mixed and centrifuged. RNA in the final aqueous phase was precipitated by the addition of 2.5 volumes of cold 95% ethanol and stored at -20°C . RNA was pelleted (14,000 g $\times$ 10 min), washed in 66% ethanol containing 0.03 M NaCl, repelleted, dried under a stream of N_2 and taken up in water and frozen in small aliquots.

Poly (A)-containing RNA was prepared by two cycles of oligo (dT)-cellulose chromatography as described by Bantle et al. (5). RNA samples were adjusted to High Salt Buffer (HSB 0.5 M NaCl, 0.01 M Tris, pH 7.5, 0.001 M EDTA) and applied to 0.5-1.0 g oligo (dT)-cellulose (Collaborative Research, type T-3) packed in a small column. Elution from the column was monitored at 254 m μ . The column was then washed with HSB until the breakthrough material, containing non-poly

(A)-containing RNA was washed through. The column was then washed with Intermediate Salt Buffer (ISB, same as HSB except with 0.1 M NaCl) and finally poly (A)-containing RNA was eluted in Low Salt Buffer (LSB, 0.01 M Tris, 0.001 M EDTA). This first pass poly (A)-containing RNA was ethanol precipitated and dissolved in 0.1 M LiCl, 0.005 M EDTA, 0.01 M Tris, pH 6.5, and 82% dimethyl sulfoxide. RNA thus treated was heated to 55°C for 5 minutes, diluted with 10 volumes 0.5 M NaCl and rechromatographed on oligo (dT)-cellulose as before. Treatment of the first poly (A)-containing fraction with dimethyl sulfoxide prior to the second cycle was essential for complete elimination of rRNA from the final poly (A)-RNA fraction. The poly (A)-RNA was concentrated to 0.2 A₂₆₀/ml by lyophilization, and was then precipitated by adjusting the solution to 0.2 M NaCl and adding 2.5 volumes of 95% EtOH. The precipitate was recovered by centrifugation, dried, dissolved in water, and frozen in small aliquots at -70°C.

For analysis by SDS-sucrose density gradient centrifugation, RNA samples were heated to 65°C for 30 seconds, cooled to 0°C, and layered onto 15-30% sucrose gradients made up in 10 mM Tris, pH 7.2, 1 mM EDTA and 0.1% SDS. The gradients were centrifuged at 50,000 rpm for 4 hours at 3°C, in a SW 50.1 rotor, collected by displacement with 40% sucrose and monitored (ISCO UA-5) optically during the collection. Fractions (approximately 0.25 ml) were adjusted to 0.1 M NaCl and precipitated by the addition of 2.5 volumes of 95% EtOH in the presence of E. coli tRNA (0.2 A₂₆₀/ml), collected, dried, and redissolved in H₂O, as described above.

Cell-Free Translation of RNA

An S-30 wheat germ lysate (100-115 A_{260} /ml) was prepared as described (73) and stored in 0.2 ml aliquots in liquid nitrogen. (Wheat germ was purchased from General Mills, Vallejo, California). Translation assays (50-200 μ l) were made up as follows: 24 mM Hepes, pH 7.6, 100 mM KCl, 2.5 mM $MgAc_2$, 1 mM DTT, 6 mM creatine phosphate, creatine phosphokinase (200 μ g/ml), 0.88 mM ATP (Sigma, neutralized), 0.018 mM GTP (Sigma), 19 amino acids (minus leucine) (20 μ M), 8 μ M [^{14} C] leucine (Amersham-Searle, 324 mCi/ μ mole) wheat germ S-30 (200 μ l/ml) and 30 μ g/ml spermine (Sigma). The reaction mixture was incubated for 90 minutes at 25°C. Following incubation, the reaction mixture was centrifuged at 40,000 rpm $\times$ 1 hr in the type 65 rotor at 2°C, and the ribosomal pellets were either discarded or used as indicated. To analyze for incorporation of [^{14} C]leucine into total protein, aliquots (25-75 μ l) of the supernatant were diluted to 0.5 ml in buffered saline containing 10 mM DL-leucine and precipitated with an equal volume of 10% TCA also containing 10 mM leucine. After 30 min at 0°, 1 ml of 5% TCA was added to the mixture and incubated on ice for an additional hour. The precipitate was collected by centrifugation (14,000 $\times$ g for 10 min, in a Sorvall HB-4 rotor), resuspended in 5% TCA, 10 mM leucine, and pelleted. The final pellet was washed with ether, dried, and dissolved in 1% SDS, 0.01 M DTT at 100°C for 3 min. An aliquot of the sample was removed for scintillation counting, and the remainder was analyzed by SDS-polyacrylamide gel electrophoresis (125).

Incorporation of radioactive amino acid into albumin was determined by immunoprecipitation, which was done by adding the following reagents

to an aliquot of the wheat germ assay supernatant, to give a final volume of 1 ml: monospecific anti-albumin (190 μ g); carrier MSA (10 μ g); Triton X-100, 0.4% (w/v); sodium deoxycholate 0.4% (w/v); and phosphate buffered saline (PBS) containing 10 mM leucine. The reaction mixture was incubated at 37° for 1 hr and at 4°C overnight, and the precipitate was collected by centrifugation (2500 rpm $\times$ 10 min), washed twice in PBS-10 mM leucine, once with Triton X-100 and sodium deoxycholate and once without the detergents. The final immunoprecipitate was dissolved and processed for SDS-polyacrylamide gel electrophoresis and counting as described above for the TCA precipitated sample. Electrophoresis was performed in 6 $\times$ 80 mm cylindrical gels of 10.5% polyacrylamide containing 0.1% SDS at 3 ma/gel for 90 min. After electrophoresis, gels were sliced, dissolved in H_2O_2 , and counted. Molecular weight markers, run on separate gels, were stained and scanned: mouse serum albumin (m.w. 68,000), A; ovalbumin (m.w. 43,000), O; chymotrypsinogen (m.w. 23,000), c; bromophenol blue, BPB.

III. RESULTS

Immunoprecipitation of Albumin Synthesizing Polysomes

Analytical small-scale direct immunoprecipitations were performed in order to determine optimal conditions for the immunoprecipitation of albumin synthesizing polysomes. The technique used was a modification of the method described by Schechter (122) for the indirect immunoprecipitation of immunoglobulin-synthesizing polysomes. In Figure III-1 are summarized the results of analytical precipitation of albumin polysomes. For each concentration of antibody tested, carrier albumin

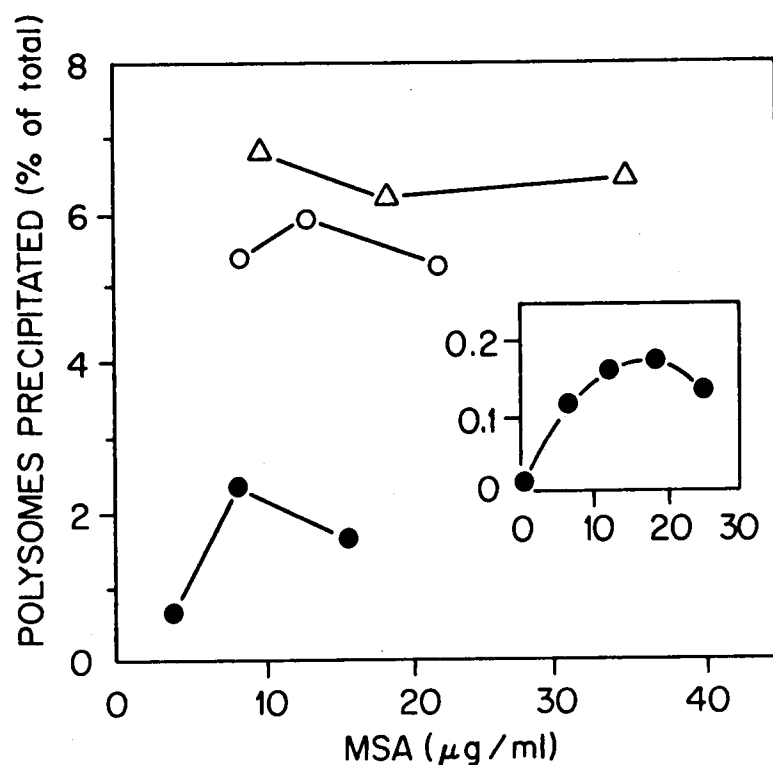


Figure III-1. Immunoprecipitation of albumin synthesizing polysomes with varying antibody concentrations.

Conditions of immunoprecipitation were as described in Experimental Procedures. Final antibody concentrations used were: 180 $\mu\text{g/ml}$ ($\bullet$ — $\bullet$); 360 $\mu\text{g/ml}$ ($\circ$ — $\circ$); 510 $\mu\text{g/ml}$ ($\triangle$ — $\triangle$). The inset shows the precipitation curve which characterizes the antibody used throughout this study. The final concentration of antibody was 180 $\mu\text{g/ml}$ and the A_{280} values (ordinate) represent the absorbance at 280 nm of the washed immunoprecipitate dissolved in 1.0 ml of 0.1 N NaOH.

was added in increasing amounts, always maintaining, however, a condition of antibody excess. It may be seen that the highest concentration of antibody tested (510 $\mu\text{g/ml}$) precipitated no more polysomes than the intermediate concentration of antibody (360 $\mu\text{g/ml}$) or about 6-7% of the total polysome sample. The lowest concentration of antibody (180 $\mu\text{g/ml}$) was effective in precipitating no more than 2.5% of the polysomes. For the large-scale immunoprecipitation of albumin polysomes, the intermediate concentration of antibody, 360 μg antibody per ml, was chosen with a corresponding albumin concentration of 14 $\mu\text{g/ml}$.

Size of Purified Albumin mRNA

Poly(A)-containing RNA from immunoprecipitated polysomes and total liver polysomes were purified by two cycles of oligo (dT)-cellulose chromatography and sedimented on SDS-sucrose gradients (Figure III-2 (A)). The poly(A)-RNA from immunoprecipitated polysomes, which sedimented as a single peak, had a sedimentation coefficient slightly smaller than the 18 S marker, i.e., approximately 17S. This corresponds to a molecular weight of approximately 6.5×10^5 , relative to 16S and 18S rRNA (76). Based on an average of 321 g RNA nucleotides per mole, albumin mRNA contains approximately 2000 bases. The final yield of poly(A)-containing albumin mRNA was approximately 50 micrograms from 3600 A_{260} units of total liver polysomes.

Total liver poly(A)-containing RNA was also prepared by two successive cycles of oligo (dT)-cellulose chromatography. This RNA (Figure III-2 (B)) sedimented as a broad peak with an average S value

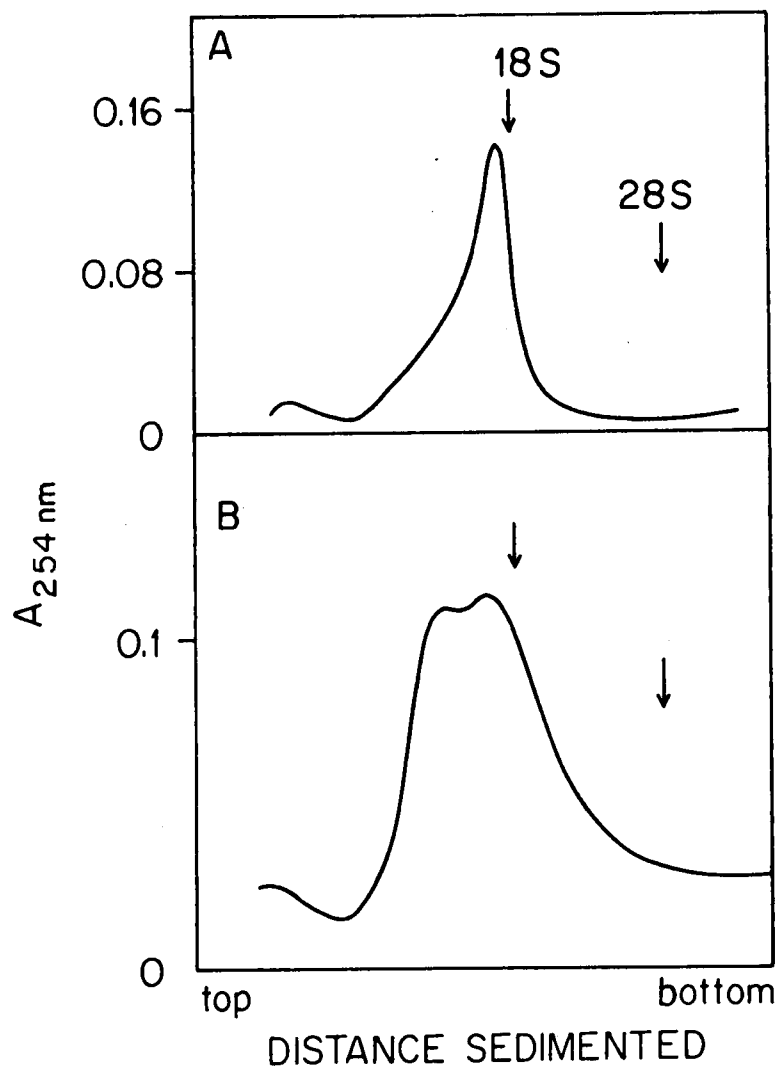


Figure III-2. Sedimentation of purified albumin mRNA and liver total poly(A)-containing RNA on SDS-sucrose density gradients.

Direction of sedimentation is left to right. Arrows indicate sedimentation of 18S and 28S RNA markers run in separate gradients. Panel A, poly(A)-containing RNA from immunoprecipitated polysomes; Panel B, poly(A)-containing RNA from mouse liver.

of 14-15S. In both cases, no 18S or 28S contaminant was observed after the second oligo (dT) treatment.

Translation of Albumin mRNA in a Cell-Free System

Characterization of the wheat germ cell-free translation system programmed with total poly(A)-RNA from mouse liver reveals that the synthesis of albumin is sensitive to KCl concentration. Optimal synthesis of immunoprecipitable albumin occurs at 100 mM KCl, whereas total amino acid incorporation and incorporation into protein released from the wheat germ ribosomes were optimal at the lowest concentration tested (Figure III-3 (A)). The data in Figure III-3 (B) show that higher concentrations of KCl favor both completion of albumin as well as release of radioactive protein from the ribosomes. However, since the translational efficiency at KCl concentrations greater than 110 mM is low, all further translation assays were carried out at 100 mM KCl.

Translation of RNA from the immunoprecipitated albumin polysomes is shown in Table III-1. Albumin synthesis in the wheat germ system increases from 4.5% of the total incorporation when programmed with total liver Poly(A)-RNA to 49.5% when programmed with RNA from immunoprecipitated polysomes indicating an approximately 11-fold enrichment of albumin mRNA. This is in good agreement with the results on the enrichment of rat albumin mRNA prepared by immunoprecipitation (141).

An SDS-polyacrylamide gel analysis of the labeled products immunoprecipitated from the wheat germ system programmed with total liver poly(A)-containing RNA is shown in Figure III-4 (A). In Figure III-4 (B) is displayed the electrophoretic profile of total TCA

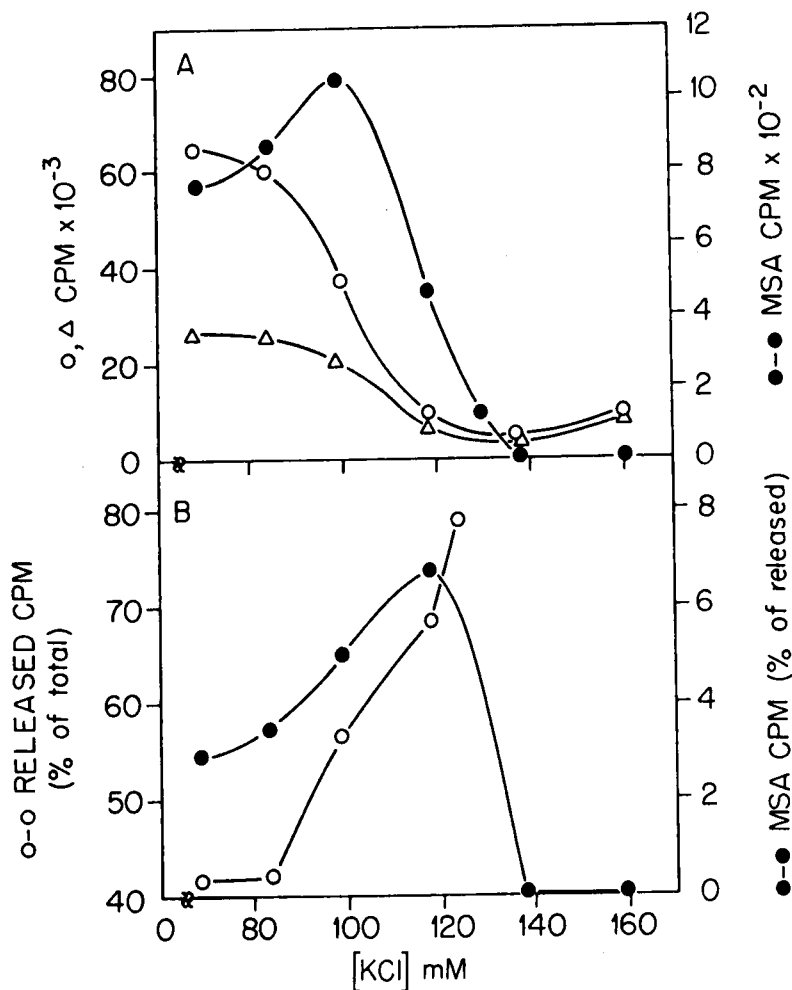


Figure III-3. The effect of KCl concentration on in vitro protein synthesis.

Cell-free translation of total liver poly(A)-containing RNA was done in the wheat germ system in a final volume of 200 μ l. Panel A, albumin synthesis (●—●); total protein synthesis representing the combined counts in pelleted ribosomes and supernatant (○—○); released counts in the supernatant alone (Δ — Δ). Panel B, albumin synthesis expressed as percentage of released counts in the supernatant (●—●); released counts as a percentage of total protein synthesis (○—○).

TABLE III-1
CELL-FREE SYNTHESIS OF ALBUMIN

RNA Sample	Total cpm/ μ g	MSA cpm/ μ g	MSA % of Total
Albumin mRNA	1,767	858	49.5
Total liver poly(A)	1,186	53	4.5

Table Legend: Purified albumin mRNA and total liver poly(A)-containing RNA after one cycle of oligo (dT)-cellulose chromatography were translated in the wheat germ system as described in Experimental Procedures. Incorporation of [^{14} C]-leucine into total released protein and into immunoprecipitable albumin are expressed per microgram of input RNA (10-20 μ g RNA per assay).

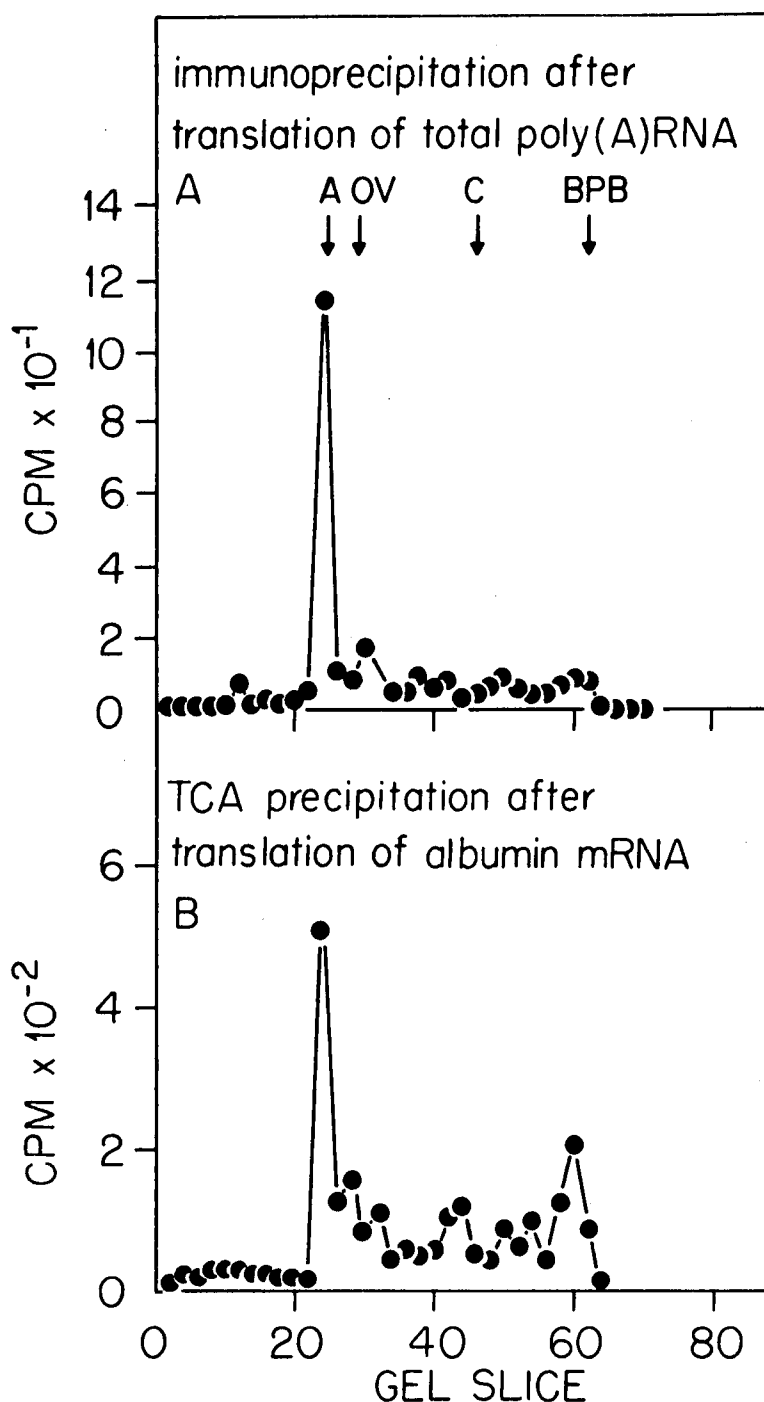


Figure III-4. SDS-polyacrylamide gel electrophoresis of the products of cell-free translation of albumin mRNA.

Panel A, albumin immunoprecipitated from 100 μ l of wheat germ reaction mixture programmed with total liver poly(A)-containing RNA; Panel B, total CCl_3COOH -precipitable products synthesized in the equivalent of 35 μ l programmed with purified albumin mRNA.

precipitated products synthesized in the wheat germ system programmed with purified albumin mRNA.

Apparent in both profiles is a single peak of radioactivity comigrating with authentic mouse serum albumin. Implications of this analysis are that the immunoprecipitation from the wheat germ lysates is specific for albumin and that the major protein synthesized under the direction of albumin mRNA is a molecule with the apparent molecular weight of mouse serum albumin. Premature termination of translation in the wheat germ system has been described and probably accounts for most of the smaller peptides seen in Panel (B) (8, 146). We could not detect preproalbumin as described by Strauss et al. (135), probably because of the relatively low resolution afforded by external protein standards and because of the large (2 mm) slices taken for analysis.

Analysis of Albumin mRNA by Sucrose Density Gradient Sedimentation and Translation of Selected Fractions

Albumin mRNA extracted from immunoprecipitated polysomes was analyzed by SDS-sucrose density gradient sedimentation in order to determine whether any contaminating mRNAs could be detected on the basis of size differences. Since no preliminary sizing of albumin mRNA was employed in purification and since the average size of liver poly(A)-RNA is considerably smaller than that of albumin mRNA (Figure III-2), any contaminating mRNA species in our preparation of albumin mRNA should be smaller than 17S, the observed size of albumin mRNA.

Fractions from an SDS-sucrose gradient were collected and assayed in the wheat germ system for their translational capacity, i.e., both total protein synthesis (TCA precipitation) and immunoprecipitation

with anti-albumin. The patterns in Figure III-5 show that both translational activities follow, symmetrically, the A_{260} nm peak which represents 17S-18S albumin mRNA.

Since the profile for total amino acid incorporation follows the A_{260} pattern and the profile of immunoprecipitable counts, we conclude that the albumin mRNA used in these studies is homogeneous in terms of size (17-18S) and in terms of function (translatability). The co-purification of heterologous mRNA species the same size as albumin mRNA, while not ruled out, seems unlikely for the reasons mentioned above.

IV. DISCUSSION

Initially, the indirect precipitation of albumin-synthesizing polysomes was attempted (126). This is an immunochemical "sandwiching" technique in which polysomes are first incubated with rabbit anti-albumin. The addition of heterologous goat anti-rabbit antibody results in the precipitation of the first antibody which has bound nascent polysomal albumin chains. Albumin mRNA, in theory, should be the only poly(A)-containing species in the resulting precipitate. Unfortunately, this technique proved unfruitful because of the nonprecipitability of the goat antibody during the short incubation times demanded by the technique.

The direct precipitation of albumin synthesizing polysomes, an antecedent to the indirect method, proved successful (94). In this technique, total polysomes are supplemented with carrier albumin and purified albumin antibodies sufficient to precipitate the added carrier.

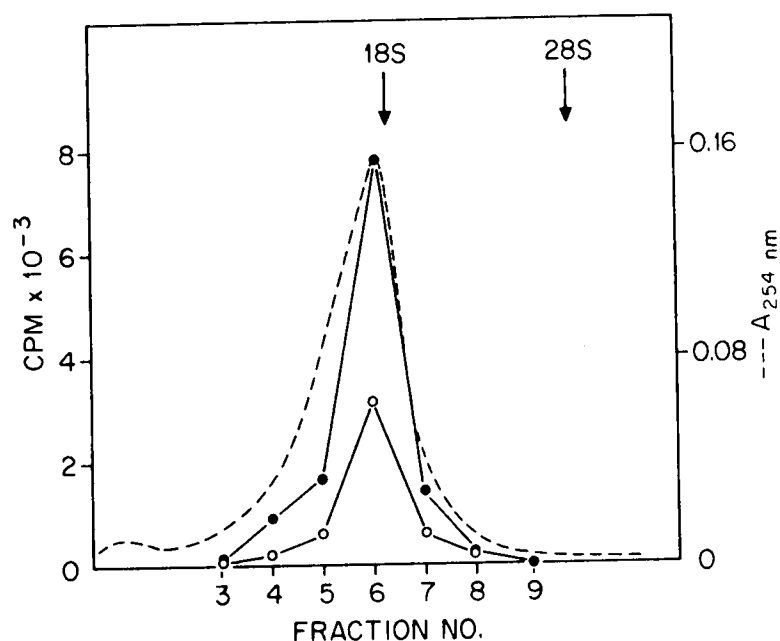


Figure III-5. Cell-free translation of albumin mRNA fractionated by SDS-sucrose density gradient sedimentation.

Albumin mRNA was sedimented through 15-30% SDS-containing sucrose gradients. Fractions were taken from the gradient and translated in the wheat germ system after which both total CCl_3COOH -precipitable radioactivity (●—●); and immunoprecipitable albumin (○—○) were determined.

Large, insoluble matrices consisting of carrier albumin, antibody, and nascent polysomal albumin are formed during the incubation and subsequently isolated by low speed centrifugation. Again, in theory, albumin mRNA should be the only mRNA in such precipitates. A major disadvantage of the direct method is that it requires copious quantities of highly purified antigen and monospecific antibody, both RNase-free. Because these are relatively easy to obtain in the case of albumin, direct precipitation was a reasonable approach. For more esoteric proteins, however, the indirect technique with superior goat antibody is the method of choice.

The main problem encountered in the immunological precipitation of specific polysomes is nonspecific adsorption and trapping of heterologous polysomes which results in contamination of the final mRNA preparation by unwanted species. Shapiro et al. (126) have shown that this nonspecific precipitation is directly related to the size of the immunoprecipitate formed during the isolation procedure. The size of the precipitate formed in the direct precipitation of albumin polysomes in this study was kept as small as possible, in order to minimize this problem as well as conserve immunological reagents.

The size of albumin mRNA is approximately 17S. This estimate is based on the sedimentation profile of purified albumin mRNA in SDS-containing sucrose gradients and on the translation of fractions taken from such a gradient. In the next chapter, these results are supported by the predominance of albumin mRNA sequences sedimenting at about 17S in totally denaturing, formamide gradients. Relative to 16S and 18S rRNA, 17S RNA should have a molecular weight of about 6.5×10^5

approximately 2000 nucleotides in length. Mouse serum albumin, molecular weight 68,000, is composed to about 580 amino acids which would require a minimum of 1740 bases for translation. If preproalbumin is the primary translation product in mouse as in rat (135), then 54 more bases are needed to code for the 18 amino acid "signal piece" required for proper membrane attachment of albumin polysomes (11). The remaining 200 or so bases could easily be accounted for by 3' noncoding and polyadenylate sequences and by 5' sequences not translated, but required for ribosome binding and initiation of translation (14, 28). In summary, mouse albumin mRNA appears to be very close in size to the minimal size dictated by translational and structural considerations common to other characterized mRNAs.

That extensive noncoding regions are not apparent deserves mention at this time. In Table III-2 are listed some recently characterized mRNAs. Estimates of size vary, due to the analytical system used to characterize the mRNA. For example, denaturing versus nondenaturing; gel electrophoresis or sedimentation. However, the four egg white protein mRNAs, all sized under identical denaturing conditions on methyl mercury agarose gels, show variability with respect to the length of the noncoding regions and the proportion of the complete mRNA that the noncoding region comprises. From this evidence, it appears that no generalizations, short of lower size limits, should be made regarding the size of eukaryotic mRNAs. The function of these noncoding regions, including the 3' poly(A) regions, remains unknown.

Short of actual sequencing data, there appears to be no single, unequivocal way to assess the purity of a specific mRNA (136). Translation

TABLE III-2
NONCODING REGIONS OF CHARACTERIZED MESSENGER RNAs

mRNA ^a	Coding ^b	Excess Bases ^c	Percent ^d
Ovalbumin (21)	1100	900	45
Conalbumin (21)	1800	950	35
Ovomucoid (21)	627	220	26
Lysozyme (21)	441	220	33
α -fetoprotein	1850	800	30
Vitellogenin (30)	6200	600	9
Immunoglobulin light chain (130)	700	500	42
Globin (107)	421	227	35
Albumin (19, 141)	1740	440, 250	20, 13

^aReferences given in parentheses.

^bMinimum bases required for synthesis of the complete protein.

^cExcess bases apparent in mRNA which codes for that protein.

^dExcess bases as a percent of the total mRNA.

in a messenger-dependent cell-free system may provide one indication of purity which, when considered with other independent methods such as nucleic acid hybridization analyses, furnish a reliable estimate of purity. Total liver polysomal RNA and albumin mRNA were translated in the wheat germ system. This system has the advantage of convenience, low expense and, most importantly, negligible background messenger activity. It is truly a messenger-dependent system as opposed to other systems derived from reticulocytes, ascites cells or brine shrimp.

Immunoprecipitable albumin was increased 11-fold relative to total leucine incorporation in the translation products programmed with purified albumin mRNA as opposed to total liver mRNA. Since albumin comprises 8-10% of total liver protein synthesis (35, 105), then one would predict a 10-12-fold increase in the in vitro synthesis of albumin with purification. Implicit in this prediction is that all liver mRNAs are translated with equal efficiency in vivo. The 11-fold purification achieved is essentially identical to that predicted. On this basis, one might conclude that the purity of the albumin mRNA preparation approaches 100%. A more conservative estimate, based on nucleic acid hybridization data, is 80-85% purity (Chapter IV).

The wheat germ system characteristically produces polypeptide fragments from premature termination of translation (8, 146). The relative inefficiency of the system in this study (no more than 50% precipitation of albumin in the products programmed with purified mRNA) probably arise from this fact. Albumin fragments could be produced which lack the antigenic determinants of the intact molecules from which the precipitating antibody was made. The gel analyses of the total

products synthesized under the direction of albumin mRNA indicate, in addition to the albumin peak, a spectrum of polypeptides ranging down to about $5-10 \times 10^3$ daltons.

Furthermore, the translation of albumin mRNA requires a salt concentration significantly different from that required for total amino acid incorporation (Figure III-3 (A), page 36). Using the same total liver mRNA preparation, relative albumin synthesis varies as much as 100% (from about 3% to 7% of total synthesis) with different salt concentrations. Absolute estimates of purity such as "percent of total synthesis" appear meaningless because of this relationship between efficiency of translation and experimental conditions. The fact that larger messengers are translated more efficiently at higher salt conditions also raises the possibility that all messengers are not alike with respect to conditions for their optimum translation (8). A plausible explanation for these salt effects is that low salt conditions favor initiation of translation, whereas ribosome translocation is depressed. Premature termination would result with many incomplete polypeptides associated with polysomes and a significant proportion of artificially released polypeptide fragments. Under high salt conditions, the converse may be true. Initiation is depressed and efficient translocation and proper termination with release favored. For practical considerations, experimental salt conditions are chosen which comprise these opposing effects.

Finally, evidence for the size homogeneity of the preparation of albumin mRNA comes from the translation of sucrose gradient fractionated mRNA (Figure III-5). The profiles of total incorporation, incorporation

into albumin, and RNA mass (A_{260}) sedimentation are essentially superimposable. This indicates that the purified preparation appears to be uncontaminated by significant amounts of bulk liver mRNA sedimenting at 14S-15S. Albumin mRNA appears from these studies to be of sufficient purity to be used for reverse transcription into complementary DNA (cDNA) and subsequently quantitation of albumin mRNA by nucleic acid hybridization.

CHAPTER IV

SYNTHESIS AND CHARACTERIZATION OF COMPLEMENTARY DNA AND THE QUANTITATION OF ALBUMIN mRNA BY NUCLEIC ACID HYBRIDIZATION

I. INTRODUCTION

Through the quantitation of specific mRNAs, a partial understanding of the molecular events of eukaryotic gene expression may be achieved. Initial attempts to quantitate specific mRNA species were by assay in heterologous cell-free translation systems such as those derived from reticulocytes (130), Kreb's ascites cells (4), bacteria (69), wheat germ (77) and brine shrimp (50). A second approach was by injection into frog oocytes (43). In all of these studies, the specific mRNA was quantitated by assessing the incorporation of labeled amino acids into the protein coded for by the mRNA under study. For mRNAs whose purification is not yet possible, this remains the only feasible way to quantitate the message. An obvious reservation in the employment of this technique is that the systems measure only functional, i.e., translatable, mRNA. The possibility always exists that mRNA is present in an untranslatable or "masked" form. Furthermore, the accuracy and reproducibility of these systems can be a problem.

With the discovery of viral reverse transcriptase (RNA-dependent DNA polymerase) came the methods to synthesize stable and highly radioactive DNA molecules which are complementary to purified mRNAs.

Because of the ability of these probes to specifically hybridize to the RNA from which the DNA was synthesized, mRNA sequences could be quantitated. In general, these hybridizations are of two types. The first type is an RNA titration in which RNA containing an unknown amount of a specific mRNA is hybridized with an excess of cDNA to saturation values. By comparing the extent of hybridization at saturation with a standard curve of known amounts of pure mRNA hybridized to cDNA, the amount of specific mRNA in the sample may be assessed (49).

In the second type of hybridization, the kinetics of hybrid formation are used to estimate the specific mRNA content of the total RNA sample hybridized. When a specific mRNA hybridizes to its cDNA, the reaction is second order and is described by the equation:

$$\frac{dD}{dt} = -kDR \quad (1)$$

where D is the concentration of unhybridized, single stranded cDNA (in moles nucleotide liter), R is the unhybridized RNA concentration (also in moles nucleotide liter) and k is the rate constant of hybridization. When RNA is in excess, the reaction is pseudo-first order with respect to cDNA and changes in RNA concentration may be disregarded. Rearrangement and integration results in:

$$\ln \frac{D}{D_0} = -kR_0t \quad (2)$$

$$\frac{D}{D_0} = e^{-kR_0t} \quad (3)$$

where D_0 and D represent the single stranded cDNA concentrations at zero time (D_0) and at any subsequent time (D) and R_0 is RNA concentration

at zero time. The extent of hybridization is provided by the ratio D/D_0 and is directly related to the product of specific RNA concentration (R_0) and time (t) of hybridization. Simply stated, a given extent of hybridization will be achieved at a given Rot value. Conventionally, the $Rot_{1/2}$ value (the Rot value corresponding to 50% hybridization, $D/D_0 = 0.5$) is used to characterize a given hybridization (10).

In most instances, the RNA hybridized to cDNA will not be a pure species but rather a heterogeneous population, such as total cell RNA, which includes the specific RNA in relatively small amounts. In these instances, the hybridization will proceed in strict accordance with the concentration of that specific RNA in the hybridization and time, as described in Equation 2. The theoretical $R_{0\text{specific}} t_{1/2}$ value (specific RNA concentration $\times$ time at 50 percent hybridization) is characteristic for that specific system under study and is unchanged from the $Rot_{1/2}$ of the purified RNA hybridized to its cDNA. However, since $R_{0\text{specific}}$ is only a minor component of $R_{0\text{total}}$ ($R_{0\text{total}} = R_{0\text{specific}} + R_{0\text{non-specific}}$), the $R_{0\text{total}} t_{1/2}$ value used to characterize the actual hybridization is increased from the theoretical $R_{0\text{specific}} t_{1/2}$ value in direct proportion to the excess, nonspecific RNA in the total sample. As a result, for various RNA samples differing in specific RNA content, increased $Rot_{1/2}$ values are related to decreased concentrations of specific RNA per unit of total RNA analyzed. Reciprocals of $Rot_{1/2}$ values, therefore, are directly related to specific RNA concentration per unit of total RNA. Additionally, the fractional purity of a given RNA (the fraction of total RNA that is a specific RNA) is given by the

relationship in Equation 4. Normally, the fractional purity of the pure RNA is taken as 1.0 (100% purity).

$$\frac{\text{fractional purity total RNA}}{\text{fractional purity pure RNA}} = \frac{\text{Rot}_{1/2} \text{ pure RNA}}{\text{Rot}_{1/2} \text{ total RNA}} \quad (4)$$

Experimentally, the extent of hybridization has been determined by hydroxyapatite chromatography (143) and, most extensively, by single strand specific S_1 nuclease digestion (72). Under the salt and buffer conditions employed, the enzyme renders all single stranded nucleic acids (unhybridized cDNA and RNA) acid soluble and nucleic acids driven into duplex hybrids may be acid precipitated. Regarding the synthesis of high molecular weight cDNA, early efforts resulted in DNA molecules significantly shorter than template mRNA length. For example, cDNA molecules of 200-300 nucleotides were commonly synthesized from mRNA templates many times that length. Two independent, though probably related discoveries enabled the synthesis of cDNA approximating the length of template mRNA. High deoxynucleotide triphosphate concentration (800 μ M versus 50 μ M) and the inclusion of pyrophosphate (4 mM) (32, 62) have resulted in the synthesis of nearly complete cDNA copies, probably by suppressing ribonuclease activity which co-purifies with the reverse transcriptase (62). One study, in particular, shows the importance of using extensive cDNA molecules in hybridization studies (152).

In this chapter, the synthesis of high molecular weight cDNA to albumin mRNA is described. The cDNA is characterized with respect to size, hybridizability to various RNAs, and fidelity of the hybrid

formed. Albumin mRNA in liver and Hepa-2 are quantitated and the size of liver albumin mRNA is confirmed by centrifugation of liver RNA in denaturing gradients and identification of albumin sequences in various fractions by hybridization to albumin cDNA.

II. EXPERIMENTAL PROCEDURES

Cells and Cell Growth

Hepa-2 cells were grown as previously described (Chapter II). Cells were always subcultured at late log phase (after 3-4 days) and never allowed to enter stationary phase.

Synthesis of Complementary DNA (cDNA)

Conditions for the synthesis of cDNA to total mouse liver mRNA and to albumin mRNA were exactly as described by Kacian and Meyers (62). Avian myeloblastosis virus reverse transcriptase was obtained from Dr. Ray Tennant (Oak Ridge National Laboratory) and was originally obtained from Dr. J. Beard. The reaction mixture (100 μ l) contained the following components which were added in the order listed: 50 mM Tris, pH 8.3; 8 mM MgCl_2 ; 0.4 mM dithiothreitol (DTT); 50 mM KCl; 200 μ M each thymidine 5'-triphosphate, 2'-deoxyadenosine 5'-triphosphate, 2'-deoxyguanosine 5'-triphosphate (P. L. Biochemicals); 200 μ M [5- ^3H] 2'-deoxycytidine 5'-triphosphate (Schwarz-Mann, specific activity adjusted to 5 Ci/ mmol with unlabeled nucleotide from P. L. Biochemicals); 20-50 μ g/ml RNA; 45 units/ml reverse transcriptase; 3 mM $\text{Na}_2\text{P}_2\text{O}_7$. The reaction was incubated for 1 hour at 37°C and divided into four 25 μ l aliquots, each of which was adjusted to 12.5 mM EDTA, and 0.5 M NaOH in

a final volume of 200 μ l. The samples were then centrifuged through 5-20% alkaline sucrose gradients (0.2 M NaOH, 0.5 M NaCl, 0.01 M EDTA;; SW-56 rotor, 50,000 rpm for 4 hours at 20°C). Fractions (0.2 ml) were collected and aliquots of each fraction were acid-precipitated in the presence of carrier tRNA. Precipitates were collected on membrane filters S+S type BA-86, and radioactivity determined. Data from these gradients were processed using the "SLG" computer program (20) to obtain a graphical representation of sedimentation profiles and molecular weights of selected fractions. Labeled bacteriophage PM-2 DNA (m.w. 3×10^6 d, a gift of W. Carrier) was run in parallel gradients as a control for the molecular weight calculations. Selected fractions from the gradients were pooled, neutralized and desalted by passage through Sephadex G-50 (Pharmacia, column dimensions 1.4 $\times$ 30 cm) equilibrated in water. Aliquots of the breakthrough material were lyophilized to dryness and stored at -70°C. No significant diminution of size was detectable when cDNA was stored in this manner for up to 4 months.

RNA Excess Hybridizations to cDNA

Hybridizations were carried out under conditions of RNA excess using the procedure of Dr. George Keller (personal communication). The reaction was performed in 6 ml polypropylene tubes (Falcon Plastics) using 10 μ l aliquots of the reaction mixture which contained 0.025 M Hepes, pH 7.4; 0.5 M NaCl; 0.002 M EDTA; 0.1% SDS; cDNA (about 1000 cpm) and RNA as indicated. Samples were overlaid with mineral oil and incubated at 68°C for the appropriate times, removed and frozen

immediately in a dry ice-acetone slurry. Assessment of the extent of cDNA-RNA hybridization was done by digestion of all single-stranded species with S_1 nuclease (Miles). The thawing sample was supplemented with 0.4 ml of S_1 nuclease buffer (0.1 M NaOAc, pH 4.9; 0.1 M NaCl; 1 mM $ZnCl_2$) containing 20 μ g of heat-denatured mouse DNA and 10 μ g of S_1 enzyme (about 5000 units). The mixture was incubated at 37°C for 1 hr and the reaction was stopped by the addition of 2 ml of 12% TCA followed by 100 μ g of mouse DNA as carrier. TCA precipitates were collected after 1 hr at 0°C on premoistened Whatman GF/C filters. Filters were washed five times with 5% TCA, twice with ethanol:ether (1:1) and dried. The precipitates were solubilized in scintillation vials in 1 ml of 0.1 N NaOH for 1 hr at 50°C, neutralized with an equivalent HCl and mixed with 10 ml Aquasol (New England Nuclear) and counted by liquid scintillation spectrophotometry. Hybridization data are expressed as the percentage of total cDNA counts in each sample that is resistant to S_1 nuclease digestion and $R_0 t$ when R_0 equals the initial concentration of RNA in mole nucleotides per liter and t = time in seconds. A value of 321 g RNA nucleotides/mole is used to calculate R_0 . All counts were corrected for background radioactivity and intrinsic S_1 resistance of the cDNA probe (2-4%).

RNA Extraction

Total liver RNA was prepared by modification of the technique described by Ross (112). Liver suspensions were prepared by homogenization in buffer (20% w/v homogenates, 10 strokes Potter-Elvehjem homogenizer in 0.01 M Tris, pH 8.0, 0.35 M NaCl, 0.001 M EDTA).

Immediately 6 volumes of urea lysis buffer were added (above buffer with 7 M urea from Schwarz-Mann, ultrapure, and 2% SDS from BDH Chemicals) and homogenization continued 2 strokes. The lysate was combined with one volume of redistilled phenol (water saturated with 0.1% 8-hydroxyquinoline) and one-half volume chloroform:isoamyl alcohol (24:1) and shaken vigorously for 10 minutes at room temperature. The emulsion was centrifuged (2000 rpm $\times$ 2 minutes) and the aqueous phase removed and re-extracted as above. The resulting aqueous phase was extracted twice for 5 minutes each with an equal volume of chloroform:isoamyl alcohol. To the final aqueous phase was added solid CsCl (previously heat-treated to inactivate any contaminating RNase) at a concentration of 1 gram per 2.5 ml. This solution was layered over 5.7 M CsCl (2.0 ml) and centrifuged to pellet RNA (SW-50.1 rotor, 35,000 rpm $\times$ 18-20 hours, 23°C). Following centrifugation, the entire gradient was rapidly removed by aspiration and the tube inverted and the bottom portion of the tube severed. The RNA pellet in the bottom of the tube was resuspended in water, dissolved (60°C for 1 min) and precipitated overnight in 66% ethanol with 0.03 M NaCl at -20°C. The yield of RNA by this method was approximately 6 mg per gram of liver and the A_{260}/A_{280} ratio always exceeded 2.1.

For the preparation of liver cytoplasmic RNA, a 10% homogenate was prepared, as above, in 0.25 M sucrose with PB₁₀₀ (0.05 M Tris, pH 7.1, 0.025 M NaCl, 0.005 M MgCl₂ with 100 ug/ml heparin). Triton X-100 was added to 0.5% from 10% stock. After gentle repipetting, a postnuclear supernatant was prepared (2000 rpm $\times$ 5 min) and the supernatant was diluted two-fold with adjustment to 0.1 M NaCl, 0.01 M

NaCl, 0.01 M EDTA and 0.5% SDS and extracted with phenol:chloroform by the technique of Perry et al. (103), as described in the previous chapter. After ethanol precipitation, the RNA was washed twice with 3.0 M sodium acetate, pH 6.5, to remove any DNA, glycogen or tRNA (78). The yield of RNA prepared by this method was approximately 6 mg per gram of liver and the A_{260}/A_{280} ratio was about 2.1.

Cytoplasmic RNA from tissue culture cells was prepared by adaptation of the above procedure and modification of our previously-described technique (20). Hepa-2 cells (20×10^6 cells per 100 mM Petri dish) were washed twice in buffer A with heparin and scraped using a rubber policeman into a volume of 1.0 ml. The cell suspension was adjusted to 0.5% Triton X-100 and gently homogenized (Dounce homogenizer, 5 strokes, "A" pestle). A postnuclear supernatant was prepared and phenol-extracted as with the liver cytoplasmic RNA preparation. RNA yields were approximately 2 mg/ 10^8 cells from late log phase cultures ($A_{260}/A_{280} \approx 2.1$).

Hydroxyapatite Thermal Chromatography

To determine the melting temperature (T_m) of albumin mRNA-cDNA, annealing was performed in fire-sealed, siliconized glass capillaries (10 μ l) as described (49). The hybrid formed at $Rot = 6$ was expelled into 1.0 ml 0.1 M Na_2HPO_4 , pH 6.8, and adsorbed to hydroxyapatite (column dimensions 1 $\times$ 2 cm) at 60°C. Thermal chromatography was performed exactly as described by Niyogi and Thomas (90). Using a temperature-step apparatus electronically coupled to column elution rate, fractions were collected representing the column elution from a

temperature increase of 1 degree Celsius. Fractions were acid-precipitated in the presence of carrier DNA, precipitates collected on filters and radioactivity determined.

Sedimentation of RNA Under Denaturing Conditions

For the sedimentation of RNA under totally denaturing conditions, the procedure of Melli et al. (81) was used with slight modification. RNA samples in water were adjusted to 90% formamide (deionized) and centrifuged through 2-10% sucrose density gradients made in 98% deionized formamide, 0.02 M Tris, pH 8.5, 0.001 M EDTA. After centrifugation, the gradients were displaced with 15% sucrose in 98% formamide and continuously monitored at 280 nm. Fractions were pooled and ethanol precipitated overnight at -20°C (133). Precipitations were collected by centrifugation (14,000 × g for 10 min, Sorvall HB-4 rotor), washed once in 70% ethanol with 0.04 M sodium acetate, re-pelleted, dried and dissolved in water.

Electron Microscopic Analysis of cDNA

Samples of cDNA were given to Mr. David Allison of the Oak Ridge National Laboratory for electron microscopic examination and cDNA was spread onto coated grids using a modified Kleinschmidt technique (2). After platinum shadowing, electron micrographs of cDNA preparations were made using a Siemens Elmiskop model 1A electron microscope at a total magnification of 106,784 X. In parallel preparations, single-stranded circular M13 phage DNA (gift of Dr. S. Mitra) was used for a molecular weight standard. The contour lengths of cDNA and M13 DNA were determined from projections of micrographs using a planimeter. Molecular weights of randomly selected cDNA molecules were estimated by comparison of

contour lengths with the contour length of M13 DNA, the molecular weight of which is 2×10^6 .

III. RESULTS

Synthesis and Characterization of cDNA

DNA complementary to albumin mRNA and to total liver poly(A)-containing RNA were synthesized by the procedure of Kacian and Meyers (62). Based on the specific activity of [^3H]dCTP (5 ci/mmol), counting efficiencies of 15-20%, and cytidine comprising 20% of cDNA by weight, the specific activity of cDNA was about 1.6×10^6 cpm/ug. The final yield of cDNA synthesized by this technique was about 10-20% of input RNA. Obviously, higher yields could be obtained by elimination of preparative sizing steps on alkaline sucrose gradients.

In Figure IV-1 are shown the sedimentation profiles of cDNA synthesized from total liver poly(A)-containing RNA and from albumin mRNA. The calculated molecular weight of the peak fraction was reproducibly 5.7×10^5 . Pooled fractions, indicated by the brackets, have a number average molecular weight of 5.3×10^5 . These molecules are about 1700 nucleotides long and are representative of about 85% of the length of albumin mRNA (M_w dNTP = 311 g/mol). These molecular weight estimates were obtained by the "SLG" computer program used extensively in the Oak Ridge National Laboratory. The accuracy of these calculations was supported by correct estimations of the molecular weight of PM-2 DNA. For comparison, the molecular weight of cDNA to total poly(A)-containing RNA was 3.5×10^5 .

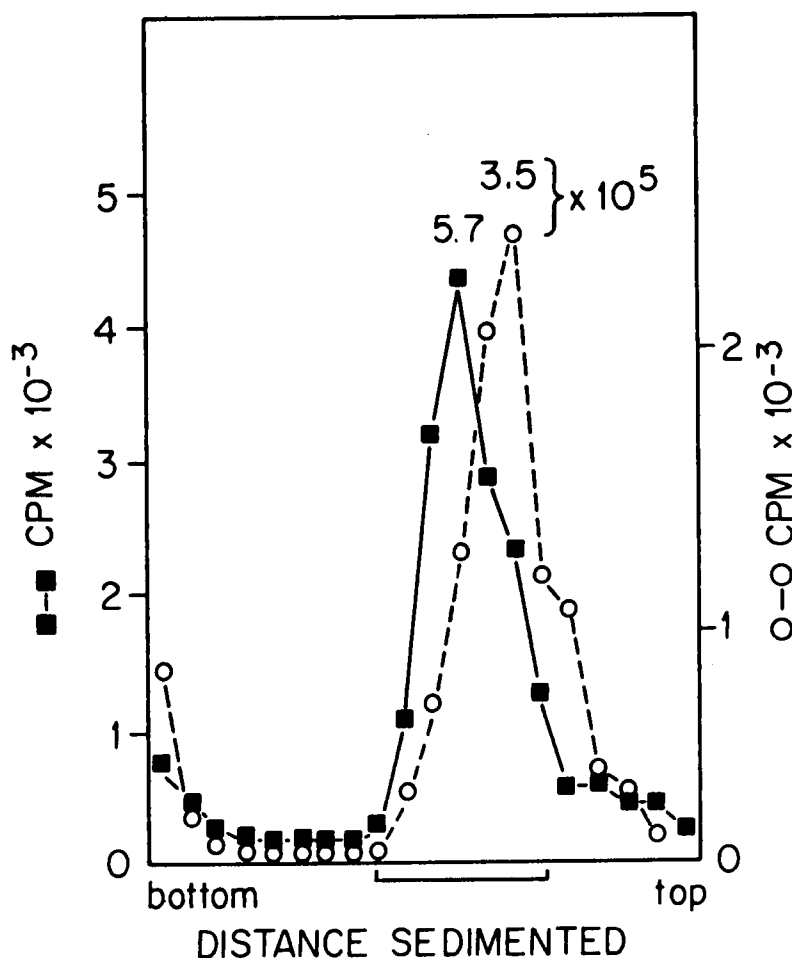


Figure IV-1. Sedimentation of albumin cDNA by alkaline sucrose density gradient sedimentation.

cDNA to albumin mRNA (■—■) and to total liver poly(A)-containing RMA (○—○) were centrifuged on 5-20% alkaline sucrose density gradients. The direction of sedimentation is from right to left. Brackets indicate fractions pooled in the preparation of albumin cDNA used in subsequent hybridization reactions.

The molecular weight of albumin cDNA was also determined by electron microscopic analysis. This method, while quite accurate for double stranded nucleic acids, tends to underestimate contour lengths of linear single-stranded nucleic acids because of the highly twisted and convoluted configurations that these molecules assume (D. Allison, personal communication). In Figure IV-2 is a histogram of the size distribution of randomly selected albumin cDNA molecules. The average molecular weight was about 4.2×10^5 (1350 nucleotides), or about 20% less than the computer-generated number average molecular weight, 5.3×10^5 . For the stated reasons, the value obtained by sedimentation is probably more accurate.

Hybridization to Albumin mRNA, Liver and Hepa-2 Cytoplasmic RNA

As an initial determination of the functionality of the probe cDNA, template albumin mRNA was hybridized, in excess, to albumin cDNA and these results may be seen in Figure IV-3. The hybridization plateaus within 100 Rot and this is an indication of a single component, pseudo-first order reaction (10). Under the salt conditions used, 0.5 M NaCl, the corresponding $\text{Rot}_{1/2}$ equals $2 \times 10^{-3} \text{ mol. sec. l}^{-1}$. Since the rate of a given nucleic acid hybridization is strictly dependent on salt concentration, hybridization parameters such as $\text{Rot}_{1/2}$ values may be corrected to standard conditions (0.12 M phosphate) by the appropriate correction factors (16). For 0.5 M NaCl, this factor is 4.24 and the $\text{Rot}_{1/2}$ value corrected to standard conditions is $8.48 \times 10^{-3} \text{ mol. sec. l}^{-1}$ ($2 \times 10^{-3} \times 4.24$). For comparison with other systems, the corrected $\text{Rot}_{1/2}$ values of ovalbumin cDNA hybridized to ovalbumin mRNA (2000

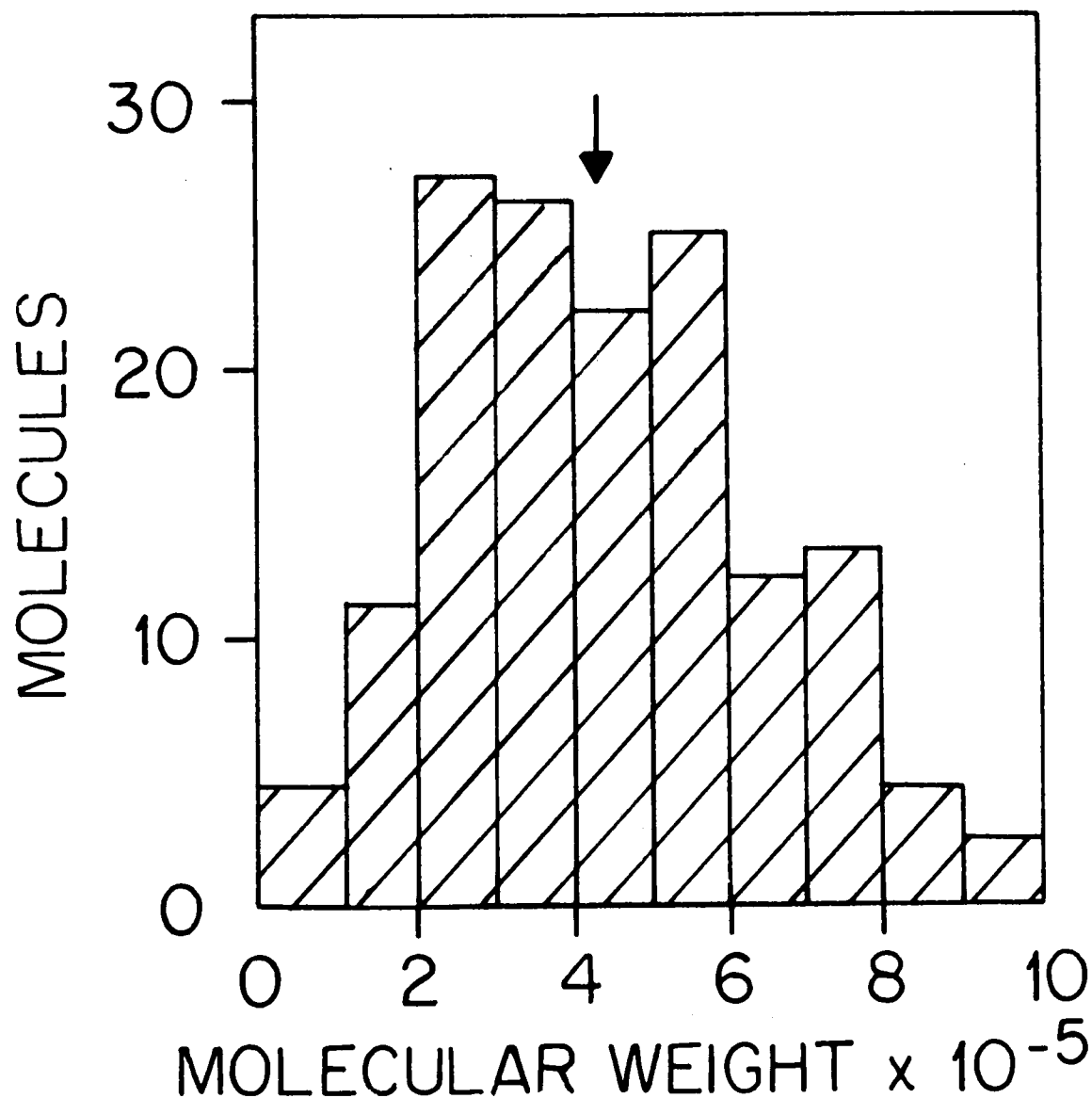


Figure IV-2. The size of albumin cDNA determined by electron microscopic examination.

Electron micrographs of albumin cDNA were prepared and contour lengths compared to that of M13 phage DNA. Molecular weights are expressed as a frequency distribution and the arrow indicates the number average molecular weight of the molecules examined.

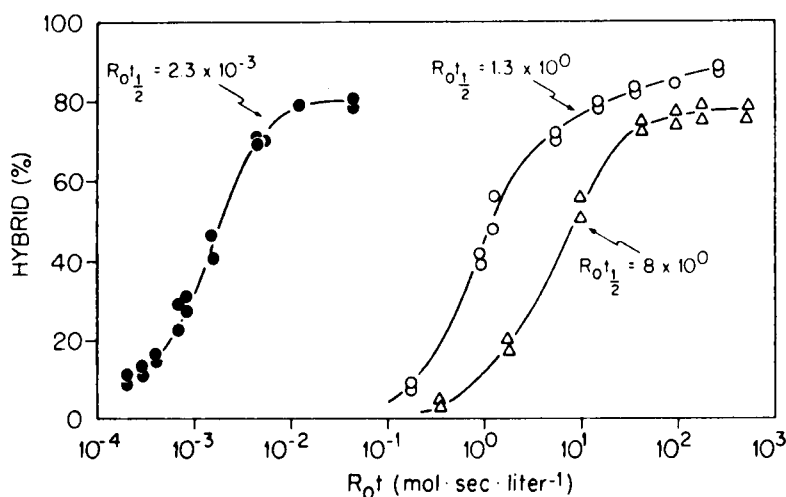


Figure IV-3. Hybridization of albumin cDNA to albumin mRNA and to cytoplasmic RNA from mouse liver and hepa-2 cells.

Albumin cDNA was hybridized to RNA and assayed by S_1 nuclease digestion. Albumin mRNA (4.4 ug/ml, ●—●) with carrier tRNA (1 mg/ml); mouse liver cytoplasmic RNA (1 mg/ml, ○—○); Hepa-2 cytoplasmic RNA (1 mg/ml △—△).

nucleotides) is 6×10^{-3} mol. sec. l^{-1} and for rat α -fetoprotein cDNA hybridized to its mRNA (2700 nucleotides), 1×10^{-2} mol. sec. l^{-1} .

Clearly, these values are very close to the $Rot_{1/2}$ for albumin mRNA-cDNA hybridization when one takes into account the experimental variation between laboratories and the higher complexity of α -fetoprotein mRNA.

Hybridization of albumin mRNA to cDNA plateaus at about 80% hybridization. This strongly indicates that the purified mRNA (and corresponding cDNA) are composed of a single species of sequences of over 80% abundance, which may be assumed to be albumin specific. The possibility does exist that the hybridization represents a multi-component reassociation with 2 or more separate species in approximately equal abundance since Rot curves are relatively insensitive in this context (10). This possibility, however, seems highly unlikely because hybridization of albumin cDNA to unrelated RNA preparations, total cytoplasmic RNA from liver and Hepa-2, produce qualitatively identical hybridizations which plateau at 80-85%. That these RNA properties (including albumin mRNA) could share major and identical abundances of nonalbumin mRNA sequences seems most improbable.

A major purpose in initiating these studies was to develop the techniques to compare and to quantitate albumin mRNA sequence levels in liver and in Hepa-2 cells. As mentioned in the Introduction, $Rot_{1/2}$ values are inversely related to the concentration of albumin mRNA in the RNA sample analyzed. As seen in Figure IV-3, accelerated kinetics are apparent in the hybridization driven by liver cytoplasmic RNA ($Rot_{1/2} = 1.3 \times 10^0$) compared to Hepa-2 cytoplasmic RNA ($Rot_{1/2} = 8 \times 10^0$). This

6-fold difference in $\text{Rot}_{1/2}$ values indicates a comparably greater albumin mRNA concentration per unit of total liver RNA than in late log phase Hepa-2 RNA. Extractable cytoplasmic RNA from liver is about 6 mg per gram of liver or about 3.5 mg per 10^8 cells (based on 1.7×10^8 cells per gram of liver (151)). In Hepa-2 cells, extractable cytoplasmic RNA is about 2 mg per 10^8 cells. Assuming equal extraction efficiencies for the two preparations, liver contains about 1.5 times more total cytoplasmic RNA than Hepa-2 cells. Albumin mRNA, therefore, appears to be 9-fold more abundant per liver cell than in Hepa-2 cells (6-fold excess per unit RNA $\times$ 1.5-fold excess of total cytoplasmic RNA).

A final calculation that may be derived from Figure IV-3 is an estimate of the number of albumin mRNAs per liver cell. A preliminary calculation is necessary to determine the fractional purity of total liver cytoplasmic RNA compared to purified albumin mRNA. The fractional purity of albumin mRNA is 0.8 (80% pure) and the $\text{Rot}_{1/2}$ values are 2.0×10^{-3} for pure albumin mRNA and 1.3×10^0 for total liver cytoplasmic RNA. Substituting into equation 4, the fractional purity of total RNA with respect to albumin mRNA is 0.00122. The following generalized equation provides an estimate of albumin mRNA per liver cell:

$$\begin{aligned}
 &(\text{cytoplasmic RNA per cell}) \times \\
 &(\text{fractional purity of RNA}) \times \\
 & (1/\text{molecular weight of albumin mRNA}) \times \\
 & (\text{Avogadro's number}) = \text{albumin mRNA/cell}
 \end{aligned}$$

Total RNA per cell is 3.5×10^{-11} gram and is based on the recovery of 3.5 mg per 10^{-8} cells; the fractional purity of total RNA is as

calculated from equation 4; the molecular weight of albumin mRNA is 6.5×10^5 grams/mole; Avogadro's number is 6.02×10^{23} molecules/mole. Substitution results in an estimate of 39,000 albumin mRNA molecules per liver cell. The corresponding value for Hepa-2 would be 9-fold less or about 4500 molecules per cell.

Thermal Denaturation of Albumin mRNA/cDNA Hybrids

The albumin mRNA-cDNA duplex formed at Rot = 6 was adsorbed to hydroxyapatite and eluted with increasing temperature while being continuously washed with phosphate buffer (Figure IV-4). Under the salt conditions employed, all double-stranded nucleic acids are selectively adsorbed to hydroxyapatite while single-stranded material is washed through. As hybrid duplexes are thermally denatured, single-stranded components are released and collected in the column eluate.

The elution profile in Figure IV-4 indicates an abrupt release of single-stranded albumin cDNA at 89°C and shows the hybrid to be extensive and characterized by a high fidelity of base-pairing. Other similar systems show comparable results (53, 84).

Size of Albumin mRNA Sequences Under Denaturing Conditions

For an independent confirmation of the size of albumin mRNA, total liver RNA was isolated and centrifuged through denaturing 98% formamide sucrose density gradients. Pooled fractions from 18 gradients were hybridized in excess to albumin cDNA in order to determine the relative albumin mRNA content of each pooled fraction. These data are represented in Figure IV-5. Hybridization data are expressed as the reciprocal of

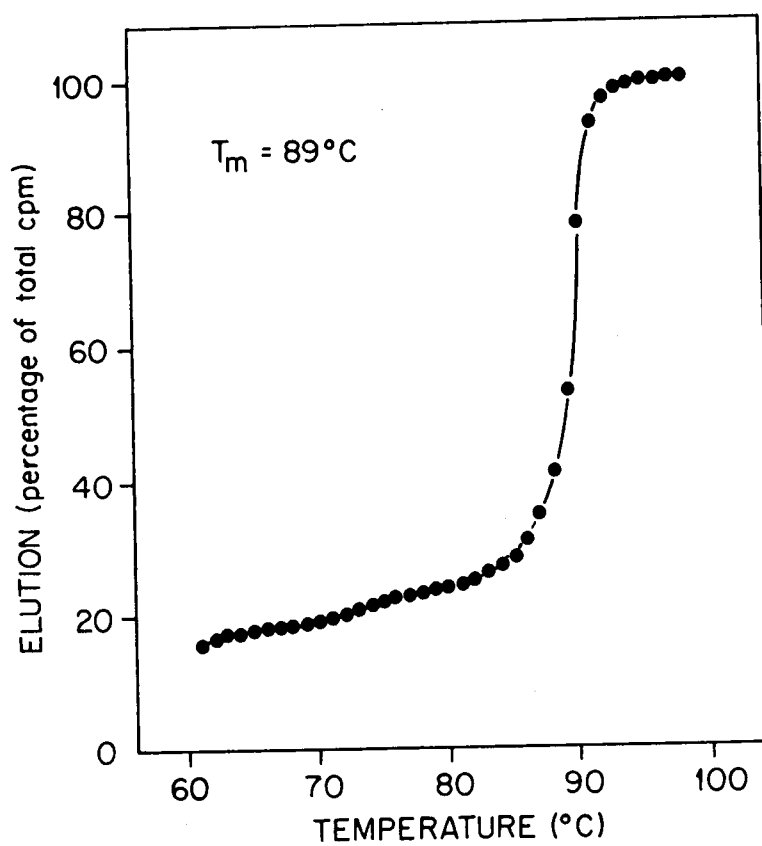


Figure IV-4. Thermal denaturation of albumin mRNA/cDNA hybrids.

The thermal chromatography of hybrid duplex was performed as described in Experimental Procedures.

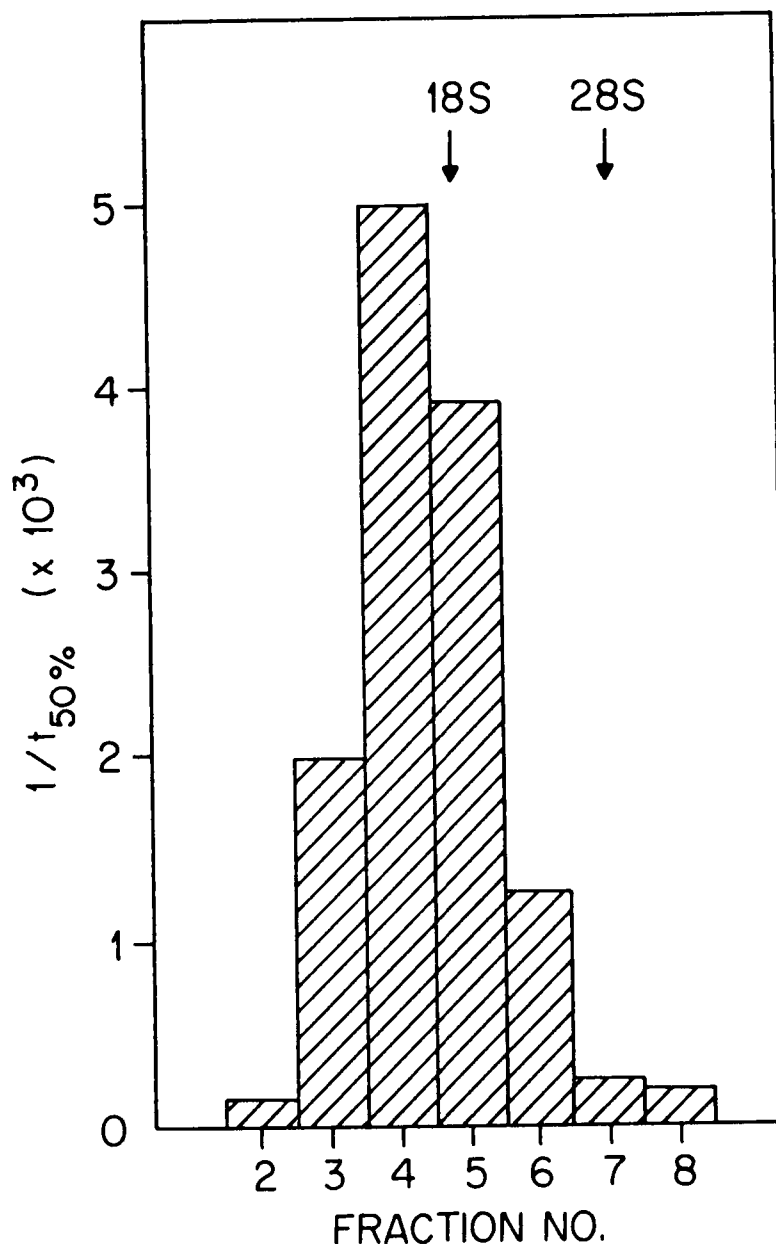


Figure IV-5. Size of albumin mRNA sequences in total mouse liver RNA sedimented in denaturing conditions.

Total mouse liver RNA (2.7 mg) was sedimented in 18 sucrose gradients containing 98% formamide and fractionated as described. RNA from each pooled fraction was rehydrated in equal volumes of water and aliquots were hybridized to albumin cDNA. The length of time necessary to reach 50% hybridization is inversely proportional to the albumin mRNA concentration of each fraction and the reciprocal of this time is represented.

time required to achieve 50% hybridization. This reciprocal value is proportional to albumin mRNA concentration in each set of hybridization and, therefore, relative albumin mRNA content in each fraction.

As seen in Figure IV-5, the bulk of albumin mRNA sequences sediment at approximately 17S under totally denaturing conditions, and this value is in excellent agreement with the size of purified albumin mRNA previously determined under nondenaturing conditions. This confirmation of size by a totally different method of RNA extraction and analysis is important in that it makes highly improbable the possibility that albumin mRNA was partially degraded during the extensive process of purification.

A second reason for undertaking these studies was to determine whether a species of albumin mRNA exists that is larger than the mature 17S molecule. Recent evidence has shown that this is the case for mRNA coding for light chain IgG (38), globin (7, 112), and histone (81). In all of these instances, the larger species was confined to the nucleus and appeared to function as a precursor to the final cytoplasmic mRNA. In the experiment summarized in Figure IV-5, it was hoped that a proposed nuclear precursor would be apparent in total RNA extracted from liver. Unfortunately, the resolution afforded by one cycle of centrifugation was probably not sufficient to clearly separate this proposed species from the mass of albumin mRNA in the cytoplasm. If fractions had been taken and re-centrifuged, it is likely that a larger species of albumin mRNA could have been identified. In a recent report, Strair et al. used this strategy and identified a nuclear 26S form of rat albumin mRNA (134).

IV. DISCUSSION

Complementary DNA was synthesized from albumin mRNA which was isolated by immunoprecipitation of albumin-synthesizing polysomes. Preparative sizing on alkaline sucrose density gradients resulted in the selection of albumin cDNA molecules which were about 85% of the length of template mRNA or about 1700 nucleotides long. Hybridization of this probe to template mRNA, to liver and to Hepa-2 RNA revealed a rapid hybridization within two Rot decades and a smooth transition to about 80% hybridization. This is interpreted as an indication that the mRNA (and corresponding cDNA) are at least 80% homogeneous with respect to albumin sequences. Thermal denaturation of albumin mRNA/cDNA hybrids resulted in a T_m (melting temperature) of 89°C indicating a well matched and extensive hybrid. By commonly accepted criteria, the albumin cDNA probe is of sufficient purity, length and functionality to be used in the quantitation of albumin mRNA sequences.

The quantitation of specific mRNA by hybridization to specific cDNA is an extremely sensitive and accurate method. Depending on the specific activity of the cDNA probe, as little as one molecule of mRNA per cell may be determined by mRNA titration methods (R. Palmiter, personal communication). By kinetic analysis, the lower limits of detection are about 10-fold higher. In consideration of the specific activity of the albumin cDNA probe and because of physical limitation on the concentration of RNA that may be reliably analyzed in solution hybridizations (5 mg/ml), the lower limits of detection for total cell

RNA are about 2000 molecules per cell and for partially purified poly(A)-containing RNA, about 50 molecules per cell.

The studies involving quantitation of mRNA by cDNA hybridization increase as the methods to isolate specific mRNA molecules of relatively low abundance improve. These include the quantitation of mRNA coding for globin (112), ovalbumin (45, 78), conalbumin (71), vitellogenin (120), α -fetoprotein (53), casein (48), light chain IgG (38), rat albumin (102) and rat α 2u-globulin (68). Using preparative hybridization techniques, relatively pure cDNA probes have been isolated from cDNA synthesized from RNA whose purity did not exceed 25% with respect to specific mRNA sequences. This technique has been used to isolate cDNA for dihydrofolate reductase mRNA (3) and rat albumin mRNA (132).

In the studies described in this chapter, albumin mRNA was found to be about 9-fold less abundant in late-log phase Hepa-2 cells compared to liver cells. In the next chapter are presented data which show that albumin synthesis is about 1% of total protein synthesis in Hepa-2 cells. In contrast, albumin synthesis comprises 8-10% of total protein synthesis in rodent liver (35, 105). It would appear that the decreased levels of relative albumin synthesis in Hepa-2 cells compared to liver are explained by correspondingly decreased amounts of albumin mRNA per cell. Data in the subsequent chapter may partially elucidate the reasons for decreased relative synthesis of albumin in tissue culture cells.

A conclusion in Chapter II was that, by antibody binding techniques, there were about 6-fold fewer albumin synthesizing polysomes in Hepa-2

cells than in liver cells. The present evidence indicates a 9-fold reduction in albumin mRNA and relative albumin synthesis in Hepa-2 cells compared to liver. It is most likely that this discrepancy arises from inaccuracies in the antibody binding techniques, stemming from nonspecific binding and corrective calculations.

A final observation deserves discussion at this time. As mentioned, a number of studies have quantitated specific mRNA sequences by cDNA hybridization. These systems are of interest because changes in the ultimate expression of certain genes, i.e., levels of certain proteins, are mediated by hormone administration or occur with development or neoplastic transformation. In none of these studies is there evidence that suggests that the presence of mRNA sequences does not correspond to the relative level of specific protein synthesis. In other words, since there is no discrepancy in relative levels of specific mRNA and corresponding protein synthesis, translational control does not appear to be a major factor in the regulation of gene expression of these representative systems. This is not to say that translational control in certain developmental systems such as the classic masked messenger of sea urchin (129) cannot be denied. However, in those systems that have been most thoroughly characterized, the levels of specific protein synthesis seem to be determined by levels of mRNA available for translation. The present studies of albumin synthesis in liver and in Hepa-2 cells support this view.

CHAPTER V

THE MODULATION OF ALBUMIN SYNTHESIS AND mRNA BY HYDROCORTISONE AND ANALOGUES OF CYCLIC AMP IN HEPA-2 CELLS

I. INTRODUCTION

As discussed in the introductory chapter, albumin synthesis is regulated by a wide variety of factors. In this study, two factors, hydrocortisone and adenosine cyclic 3':5' monophosphate (cAMP), have been selected for investigation of their effects on albumin synthesis and mRNA levels in Hepa-2 cells.

It has been shown by several groups that glucocorticoids can stimulate the synthesis of liver-specific proteins in vivo and in permanent cell lines derived from minimal deviation hepatomas. These proteins include, among others less thoroughly studied, the enzymes tyrosine amino transferase (TAT) (64, 144), tryptophan oxygenase (124), and serine dehydratase (54). In addition, the effects of hydrocortisone on the synthesis of serum albumin have been described (Chapter I). With the development of efficient translation systems came the demonstration that the mRNAs which code for tyrosine amino transferase and for tryptophan oxygenase are increased in a manner coordinate with increased enzyme synthesis (89, 109, 124). In general, two concepts which have emerged from these studies are that glucocorticoids can mediate the induction of new protein synthesis, or modulate the synthesis of

proteins which are synthesized at low basal levels, and that the increase in synthesis is accompanied by an increase in the translatable mRNA of these specific proteins. These concepts imply that the mechanism of regulation by glucocorticoids involves processes which lead to the increased accumulation of specific mRNA which ultimately results in the increased synthesis of the corresponding protein. The basic processes which might result in increased specific mRNA levels might be of two types (or a combination of each): the increase in transcription of a specific mRNA or a decrease in the rate at which this mRNA is degraded. Either process would result in the greater steady state levels of mRNA associated with the hormonally induced state.

The model of glucocorticoid action that has received the most attention is the transcriptional model, and this model is schematically represented in its simplest form in Figure V-1.

In this model, the steroid enters the cell and binds with high affinity to the specific steroid receptor protein in the cytoplasm. The binding event induces a conformational change in the receptor protein and, after a temperature-dependent activation step, the activated receptor-steroid complex accumulates in the nucleus where it associates with chromatin. In some, as yet unidentified, manner the association of these ternary complexes are thought to alter the transcription of defined portions of the genome to produce increased amounts of specific mRNA molecules. This model has recently been supported by data involving glucocorticoid-induced mouse mammary tumor virus (MMTV) RNA (108, 156). These studies, involving identification of pulse labeled MMTV RNA by nucleic acid hybridization techniques, have shown that increased

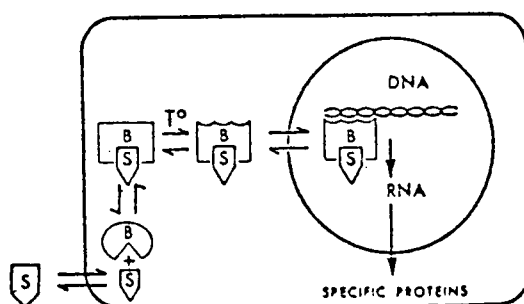


Figure V-1. Model for glucocorticoid action.

Symbols used include S, steroid; B, steroid receptor protein.
Adapted from Simons et al. (128).

synthesis results from the administration of the hormone to tissue culture cells. In looking at the degradation of MMTV RNA, one of these studies (157) seems to have ruled out the possibility that decreased degradation was responsible for the RNA accumulation since the rate of degradation was actually increased following hormone treatment. This result must be interpreted with caution, however, since actinomycin D, an inhibitor of RNA synthesis, was used. Other studies, using less direct measurements of RNA degradation, have similarly concluded that decreased degradation is not primarily responsible for increased accumulation of specific mRNAs (64). Clearly, faithful in vitro transcription of genes inducible by glucocorticoids is necessary to resolve this question.

Of crucial importance in the transcriptional model presented in Figure V-1 are the presence and specificity of receptor proteins. Where investigated, a high degree of correlation exists for the presence of specific cytoplasmic steroid receptors and biological responsiveness for sex steroids (58) and for glucocorticoids (143).

Concurrent with studies on the glucocorticoid regulation of hepatic protein synthesis have been studies which have shown that adenosine cyclic 3':5' monophosphate (cAMP) and its analogues stimulate the synthesis in vivo and in tissue culture systems of a wide variety of liver-specific enzymes (154), including tyrosine aminotransferase, phosphoenolpyruvate carboxy kinase (PEPCK) and serine dehydratase. Primarily on the basis of studies with tyrosine aminotransferase, it has been hypothesized that cAMP regulates the synthesis of this enzyme by enhancing the translation of pre-existing mRNA (154). This hypothesis

evolved because of the finding that induction of TAT by analogues of cAMP was too rapid to be explained by transcriptional modulation, as in the case for glucocorticoid induction (2-4 hours), and induction was not blocked, in early experiments, by inhibitors of RNA synthesis.

In the translational model, the specific site and mode of action of cAMP has not been identified, but protein phosphorylation catalyzed by a cAMP-dependent protein kinase was assumed to be the initial biochemical event associated with the stimulation of certain proteins. Potential sites include ribosomal subunits, mRNA associated proteins, enzymes and factors involved in initiation, translocation and termination, and even nascent polysomal peptides. In a recent report, the transit time of ribosomes engaged in the synthesis of TAT was significantly shortened by the administration of cAMP to rats (110). Recent studies, however, have seriously contested the translational model. With the use of efficient cell-free translation systems, it has been determined that the induction of TAT and PEPCK synthesis in rat liver are accompanied by proportionate increases in the functional mRNA coding for these enzymes (34, 51, 91). In consideration of these results and the results that are presented in the chapter, the proposed mechanism of cAMP action involving translation of pre-existing mRNA must be reevaluated.

In this chapter is presented evidence that hydrocortisone and cAMP analogues mediate increases in the synthesis and secretion of albumin by Hepa-2 cells and that in combination these compounds interact in an apparently synergistic manner. In addition, evidence is presented which indicates that the administration of these compounds to Hepa-2

cells result in increases in albumin mRNA sequences in direct proportion to the increases seen in albumin synthesis.

II. EXPERIMENTAL PROCEDURES

Cells and Culture Conditions

The cell line Hepa-2 was used throughout this study and was grown as previously described in Chapter IV, with the exception that insulin was excluded from the Waymouth's MAB/87/3 culture medium. No adverse effects on growth were noted from this omission. Complete media containing 1 mM $N^{6},O^{2'}$ -dibutyryl adenosine cyclic 3':5'-monophosphate (Bt_2cAMP , Sigma Chemicals, St. Louis, Mo.) or 1 mM 8-Bromoadenosine-3':5'-monophosphate (8-BrcAMP, Sigma Chemicals) were prepared daily before use; medium containing 10^{-6} M hydrocortisone (HC, Sigma Chemicals) or 10^{-6} M tetrahydrocortisone (H_4HC , Sigma Chemicals) was prepared weekly. All experiments were initiated with cells at a density of about $10-15 \times 10^6$ cells/100 mm petri dish, at which point the cells are visibly confluent. Media (8 ml) were changed at 24-hour intervals for the duration of each experiment. Control cells have a generation time of about 18 hours and reach saturation density ($40-50 \times 10^6$ cells/petri dish) within 72 hours. Cell numbers were determined with a hemocytometer.

Total Protein Synthesis and Albumin Synthesis and Secretion

Medium was removed from the cultures and replaced with 5 ml of fresh medium one hour prior to the addition of 4,5- $[^3H]$ leucine (100 $\mu Ci/5$ ml; ~50 Ci/mMole, Amersham-Searle, Arlington Heights, Illinois). For determination of rates of albumin secretion, aliquots (200 μl) of the

media were removed at various time intervals after the addition of the [^3H]leucine and frozen. Cells used for albumin synthesis and total protein synthesis were labeled as described above. At the end of the labeling period, the medium was removed and the cells were rinsed twice with ice-cold phosphate buffered saline (PBS) containing 10 mM DL-leucine, and stored at -70°C . To analyze for albumin and total protein synthesis, the cells were thawed and scraped in 2 ml PBS containing leucine. Triton X-100 was added to a final concentration of 0.5% and the suspension was vigorously homogenized (Dounce homogenizer, "B" pestle, 10 strokes). From this homogenate, an aliquot (0.5 ml) was removed for determination of total protein synthesis and the remaining sample saved for assessment of intracellular albumin synthesis.

Total protein synthesis was determined on aliquots of the initial cell homogenate which were first adjusted to 1% SDS in a volume of 1 ml. The resulting viscous solution was sheared by three passages each through a 19-gauge and 27-1/2-gauge syringe needle. An aliquot of this mixture (usually 0.1 ml) was diluted to 0.5 ml and precipitated by the addition of two volumes of 10% TCA with 10 mM DL-leucine. Carrier BSA (100 μg) was then added and allowed to incubate on ice for at least 2 hours. The precipitates were pelleted (14,000 $\text{g} \times 10 \text{ min}$) and the supernatants were discarded. The pellet was resolubilized in 0.1 ml of 0.1 N NaOH, diluted to 0.5 ml, reprecipitated as above, and finally washed with ethanol:ether (1:1). Radioactivity was determined by dissolving the pellet in 1 ml of 0.1 N NaOH and counting in Aquasol (New England Nuclear, Boston, Mass.).

For determination of intracellular albumin synthesis, the remaining initial cell homogenate was adjusted to 0.5% sodium deoxycholate, rehomogenized as above, and centrifuged ($14,000 \text{ g} \times 10 \text{ min}$). An aliquot (0.5 ml) of this supernatant was then cleared by immunoprecipitation with either rabbit anti-mouse transferrin or anti-mouse ferritin (50 μl) (Cappel Laboratories, Downingtown, Penna.) after the addition of the appropriate amount of transferrin or ferritin. The immunoprecipitate was pelleted by centrifugation ($2,000 \text{ rpm} \times 10 \text{ min}$) and discarded, and albumin (6 μg) and rabbit anti-mouse albumin antiserum (50 μl) were added to the supernatant for the immunoprecipitation of [^3H]albumin. The reaction mixture was incubated at 37°C for one hour and at 4°C overnight. The albumin immunoprecipitate was pelleted and washed twice in PBS with leucine, once with 0.4% Triton X-100 and sodium deoxycholate and once without detergents. The immunoprecipitate was prepared for either SDS-polyacrylamide gel electrophoresis (125) or dissolved in 1 ml of 0.1 N NaOH and counted by liquid scintillation spectrophotometry.

Albumin secretion was determined by immunoprecipitation of aliquots of culture medium which were removed at various times after the addition of [^3H]leucine. No prior clearing immunoprecipitation was necessary and the immunoprecipitation for albumin was performed, as described, in a final volume of 1 ml.

RNA Extraction and Hybridization

Total cell RNA was extracted by the method of Ross (112) as modified and described in Chapter IV. RNA recoveries were reproducible and varied no more than 5% on duplicate samples. RNA from as few as

15×10^6 cells has been extracted and analyzed for albumin mRNA. Hybridization of RNA to albumin cDNA under conditions of RNA excess was exactly as described in Chapter IV. Relative albumin mRNA levels are expressed as reciprocals of the $R_0 t$ values corresponding to 50% hybridization ($1/R_0 t_{1/2}$); R_0 equals the initial concentration of RNA in mole nucleotides per liter and t = time in seconds. Precise determinations of $1/R_0 t_{1/2}$ values were derived from the linear plots of the reciprocal of percent hybridization (1/percent hybridization) versus the reciprocal of $R_0 t$ (78). A value of 321 g RNA nucleotides per mole is used to calculate R_0 .

III. RESULTS

Albumin Synthesis

Preliminary dose-response experiments were performed with hydrocortisone (10^{-7} , 10^{-6} and 10^{-5} M) and dibutyryl cAMP (10^{-4} , 5×10^{-4} and 10^{-3} M treatment of Hepa-2 cells for 72 hours. From these experiments, it was determined that 10^{-6} M hydrocortisone and 10^{-3} M Bt₂cAMP produced maximal increases in albumin secretory rates relative to untreated controls (data not shown). The inclusion of insulin, a normal component of Waymouth's MAB/87/3 media (8 μ g per ml), had no effect on cell growth and little effect on albumin synthesis in control cells (15% increase). In order to eliminate any unwanted combined hormonal effects of additional insulin with hydrocortisone or Bt₂cAMP, insulin was eliminated from the growth media.

A time course of albumin synthesis as determined by rates of secretion of labeled albumin in the presence of hydrocortisone (10^{-6} M)

and Bt_2cAMP ($10^{-3}M$) is presented in Figure V-2, along with growth curves for the cells under the experimental conditions described. A comparison of hydrocortisone and Bt_2cAMP -treated cells reveals that after 72 hours of treatment, there is a 2.6-fold increase in the rate of albumin synthesis in hydrocortisone-treated cells (Figure V-2 (A)) and a 3.6-fold increase in Bt_2cAMP -treated cells relative to untreated control cells. In both cases, rates of albumin synthesis in control cells decrease approximately 50% as cells enter stationary phase. Depression of albumin synthesis in stationary phase cells has been described for the parent cell line (9) and for a rat hepatoma cell line (92).

In addition to the stimulation of albumin synthesis, both hydrocortisone and Bt_2cAMP show striking effects on the growth of Hepa-2 cells. Hydrocortisone treatment extends the generation time to about 28 hours, whereas Bt_2cAMP treatment essentially arrests cell division after 48 hours of treatment. Increased cAMP levels have classically been associated with decreased cell growth in a variety of cell types (119). Coincident with arrest of cell division Bt_2cAMP -treated cells show extensive morphological change. Within 8 hours of treatment, adherent cells, which are normally flattened and extended in appearance, become withdrawn and develop stellate processes. By 36 hours, and for the duration of the experiment, the cells are highly compacted and rounded. Similar findings have been reported by others (149).

In past studies, the assessment of rates of albumin synthesis in Hepa cells was facilitated by the fact that secretion rates after 60 minutes of labeling are linear and identical to initial rates of

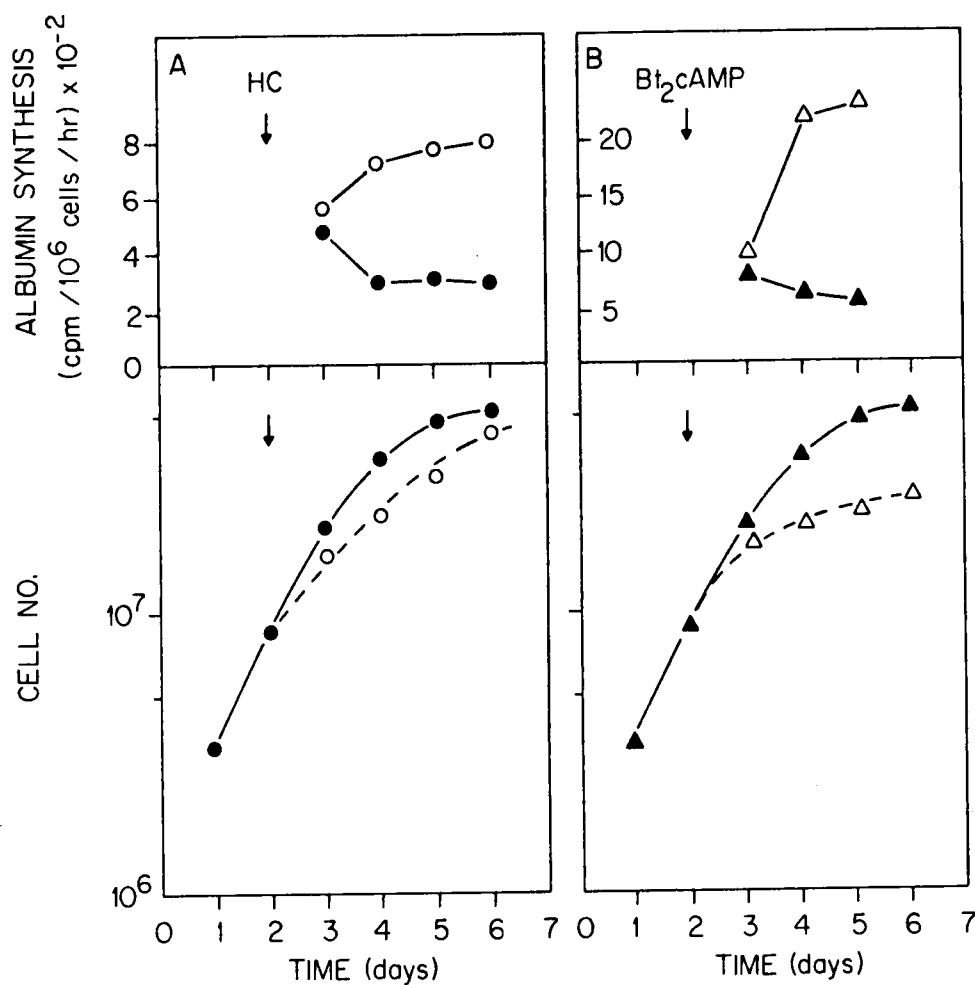


Figure V-2. The effects of hydrocortisone and dibutyryl cAMP on the rate of albumin synthesis and on cell growth.

Hepa-2 cells were grown to mid-log phase and treated with hydrocortisone in Panel A; and Bt₂cAMP in Panel B. The rates of albumin secretion were determined by labeling the cells with [³H]-leucine (20 uCi/ml) for 3 hours and immunoprecipitating albumin from the growth media at hourly intervals. Albumin secretion for untreated cells (●—●); hydrocortisone-treated cells (○—○); and Bt₂cAMP-treated cells (△—△). The arrow indicates the time of addition of either compound. The top panels of Panels A and B represent secretory rates (anti-albumin precipitable cpm/ 10^6 cells/hr); the bottom panels represent growth curves for control and treated cells.

intracellular albumin accumulation, i.e., synthesis. In the present studies, similar analyses were done to show that a direct relationship between rates of synthesis and secretion persist in hydrocortisone and in Bt_2cAMP -treated cells and that rates of secretion can justifiably be used to assess rates of synthesis. In Figure V-3 are presented data on the accumulation of labeled albumin intracellularly and in the growth media as a function of time after labeling with [^3H]leucine. Incorporation of labeled amino acid into total protein is also included. Radioactively labeled albumin can be detected within 5 minutes in Hepa-2 cells and continues to accumulate in a linear manner for about 30 minutes. After 30 minutes, accumulation slows as newly synthesized, labeled albumin is secreted and begins to accumulate in the growth media. After 60 minutes, the rate of accumulation of secreted albumin becomes linear and intracellular albumin accumulation stabilizes as steady-state levels are approached where rates of synthesis and secretion of labeled albumin are equal. From these figures, the rate of initial albumin accumulation was obtained by determining the slope of a line connecting points up to and including 30 minutes of labeling. These values are assumed to represent the true rate of albumin synthesis and are compared with the corresponding slopes of albumin secretion after 60 minutes in Table V-1.

From Table V-1, it may be seen that rates of synthesis and secretion are essentially identical and therefore may be used interchangeably. Total protein synthesis occurs linearly throughout the course of the experiment.

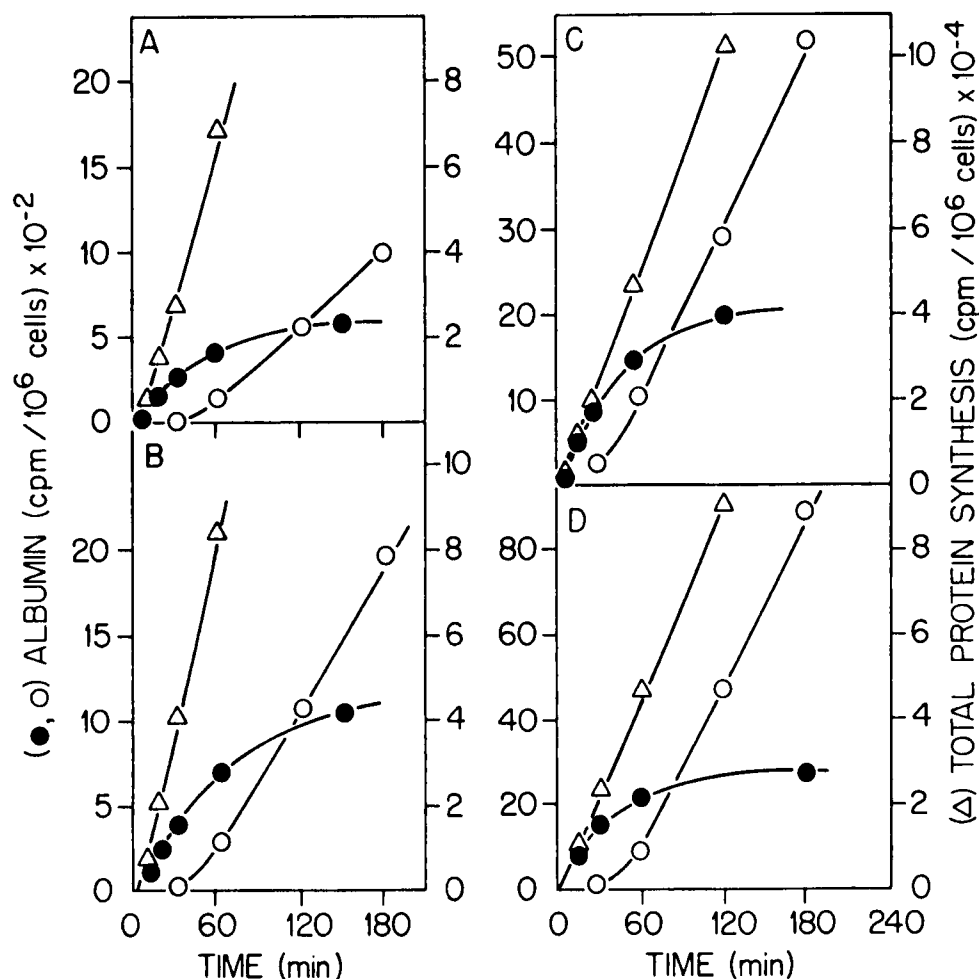


Figure V-3. The effects of hydrocortisone and dibutyryl cAMP on total protein synthesis, albumin synthesis and albumin secretion.

Hepa-2 cells were grown for 72 hours in the presence of control media, Panel A; hydrocortisone, Panel B; Bt₂cAMP; Panel C; and hydrocortisone and Bt₂cAMP, Panel D. At various times after the addition of [³H]-leucine to the culture medium, samples were taken for the determination of albumin synthesis (●—●); albumin secretion (○—○); and total protein synthesis (▲—▲). Note changes in the values of the ordinate.

TABLE V-1
COMPARISON OF ALBUMIN SYNTHESIS AND
SECRETION IN HEPA-2 CELLS

Treatment	Albumin cpm/10 ⁶ cells/min.	
	Synthesis	Secretion
None	8.7	8.6
Hydrocortisone	12.3	14.2
Bt ₂ cAMP	37.2	34.1
Hydrocortisone + Bt ₂ cAMP	52.0	66.2

From Figure V-3, the rates of albumin synthesis were determined from the slopes of the plots of intracellular albumin accumulation up to 30 minutes. Secretion rates were similarly measured from the accumulation of albumin in the media after 60 minutes of labeling.

The data in Figure V-3 indicate that hydrocortisone and Bt_2cAMP mediate increases in intracellular albumin at steady state conditions which are commensurate with increases in albumin synthesis and secretion. The time of appearance of albumin in the growth media is not appreciably changed by either compound alone or in combination, suggesting that processing and secretory functions are unaffected. Clearly, more detailed experiments are needed before this may be stated with certainty but, at this point, elevated albumin mRNA levels could explain the increased synthesis and secretion of albumin in the treated cells.

Immunochemical Identification of Intracellular and Secreted Albumin

The data on protein synthesis are only as good as the techniques used to identify newly synthesized albumin. In order to determine the specificity of the immunoprecipitations, ^{14}C -labeled secretion products from Hepa-2 cells and ^3H -labeled cell lysates were mixed and immunoprecipitated with albumin antibody as specified. The final immunoprecipitate was then electrophoresed on sodium dodecyl sulfate (SDS) containing polyacrylamide gels. In Figure V-4 are presented the results of this experiment. Both intracellular and secreted albumin migrate as single peaks of radioactivity coincident with the migration of authentic serum albumin. Thus, the immunoprecipitation technique appears to be specific for albumin and very little or no contaminating material is apparent. Furthermore, it has been shown that this procedure is capable of precipitating greater than 95% of chemically purified, labeled serum albumin (data not shown).

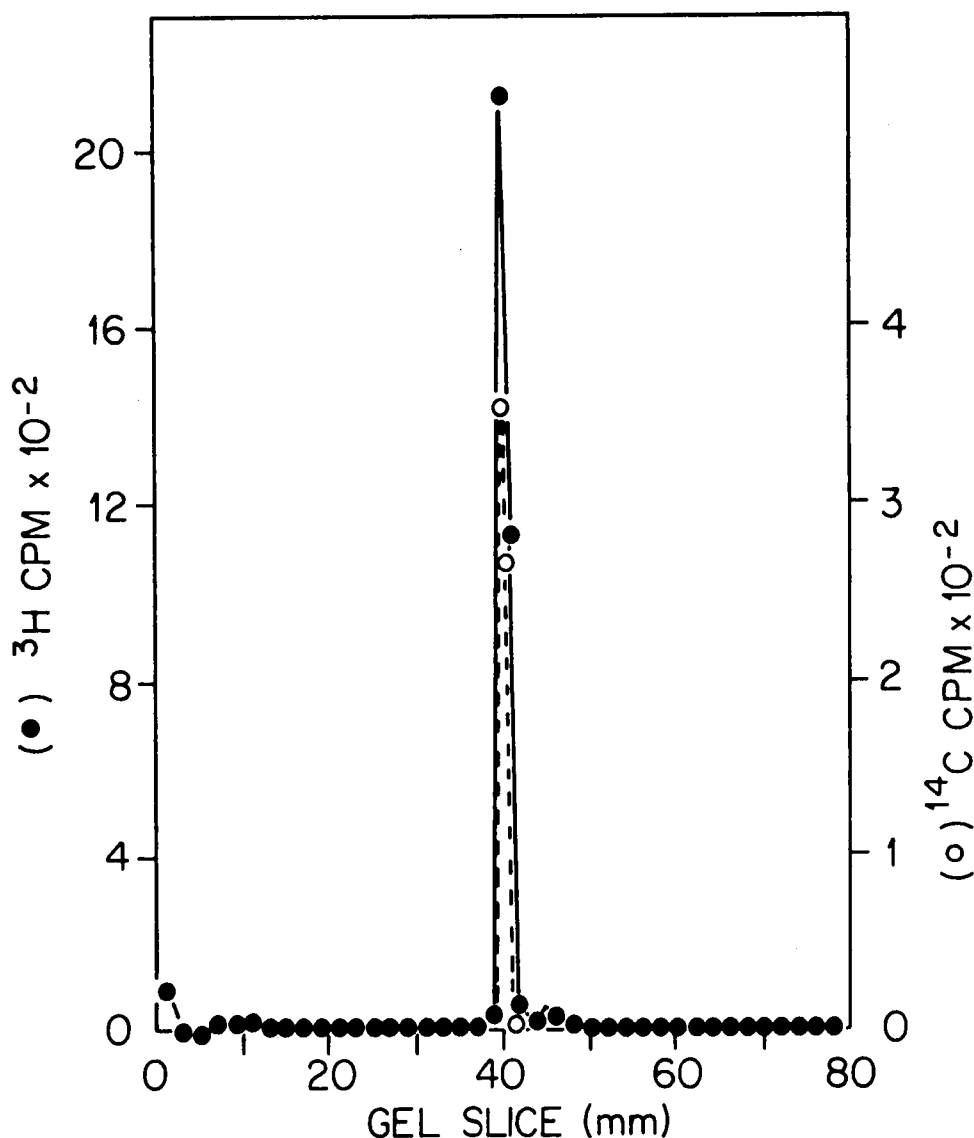


Figure V-4. SDS-Polyacrylamide gel electrophoresis of immunoprecipitated intracellular and secreted albumin.

Culture medium (100 μl) containing ^{14}C -labeled secreted proteins was mixed with a Hepa-2 cell lysate equivalent to about 1.5×10^7 cells (0.8 ml) labeled with [^3H]-leucine for 30 minutes. The mixture was processed as described for intracellular albumin in Experimental Procedures. The immunoprecipitated albumin was electrophoresed on 10.5% SDS-polyacrylamide gels. The radioactivity was corrected for spillover (23%). Intracellular albumin (●—●); secreted albumin (○—○).

A second reason for performing the experiment shown in Figure V-4 was to attempt to identify an intracellular preproalbumin species which has been seen in the cell-free synthesis of rat albumin mRNA (135). It was hoped that the precursor might be identified as a slightly larger (24 amino acids) and more slowly migrating shoulder of radioactivity in the immunoprecipitated sample from the cell lysate. Since no larger species is apparent in the gel profile, it is possible that the processing of the precursor occurs even while the protein is being synthesized and that, in vivo, preproalbumin does not exist. The gel system used should have been able to distinguish the larger molecule because α -fetoprotein, the molecular weight of which is about 70,000 daltons and approximates the molecular weight of preproalbumin, may be resolved from albumin in this system (M. Morales, personal communication). Furthermore, it is highly unlikely that preproalbumin lacks all of the antigenic determinants which are characteristic of serum albumin and it is unlikely that the antibody preparation was incapable of immunologically recognizing preproalbumin.

Quantitation of Albumin mRNA Sequences in Hepa-2
Cells After Treatment with Hydrocortisone,
Dibutyryl cAMP and 8-Bromo cAMP

In the previous chapter are described the preparation and characterization of mouse albumin mRNA and the use of complementary DNA to quantitate albumin mRNA sequences in liver cells and in Hepa-2 cells. In the present studies, kinetic analyses of albumin mRNA levels have been completed for the following: (a) total RNA from Hepa-2 cells

treated with hydrocortisone and tetrahydrocortisone (Figure V-5 (A)); (b) Bt_2cAMP and butyric acid (Figure V-5 (B)); (c) 8-bromo cAMP (Figure V-5 (C)); and hydrocortisone plus Bt_2cAMP administered simultaneously (Figure V-5 (D)).

The accelerated reassociation kinetics seen with RNA extracted from hydrocortisone and cAMP-treated cells indicate that the increase in relative rates of albumin synthesis, mediated by these two factors, may be due to the elevation in albumin mRNA levels. The specificity of both effects are indicated by the failure of both tetrahydrocortisone, a biologically inactive analogue of hydrocortisone, and butyric acid to increase albumin mRNA levels.

Figure V-5 reveals that it is difficult to accurately derive $Rot_{1/2}$ values from these plots, especially when only 2-fold differences in hybridization kinetics are apparent. Transformation of the data into double reciprocal form results in linear plots from which the reciprocals of $Rot_{1/2}$ values, which are proportional to relative albumin mRNA per unit of RNA, may be accurately derived. In all subsequent comparisons of albumin mRNA levels, the reciprocals of $Rot_{1/2}$ have been determined graphically from double reciprocal plots of 1/percent hybridization versus $1/Rot$.

In Table V-2 are presented protein synthesis data, along with corresponding albumin mRNA comparisons. Throughout the course of the study, considerable variation in basal levels of albumin synthesis and mRNA levels were apparent. The reasons for this variation are uncertain but similar fluctuations have been reported by others (55). As a result

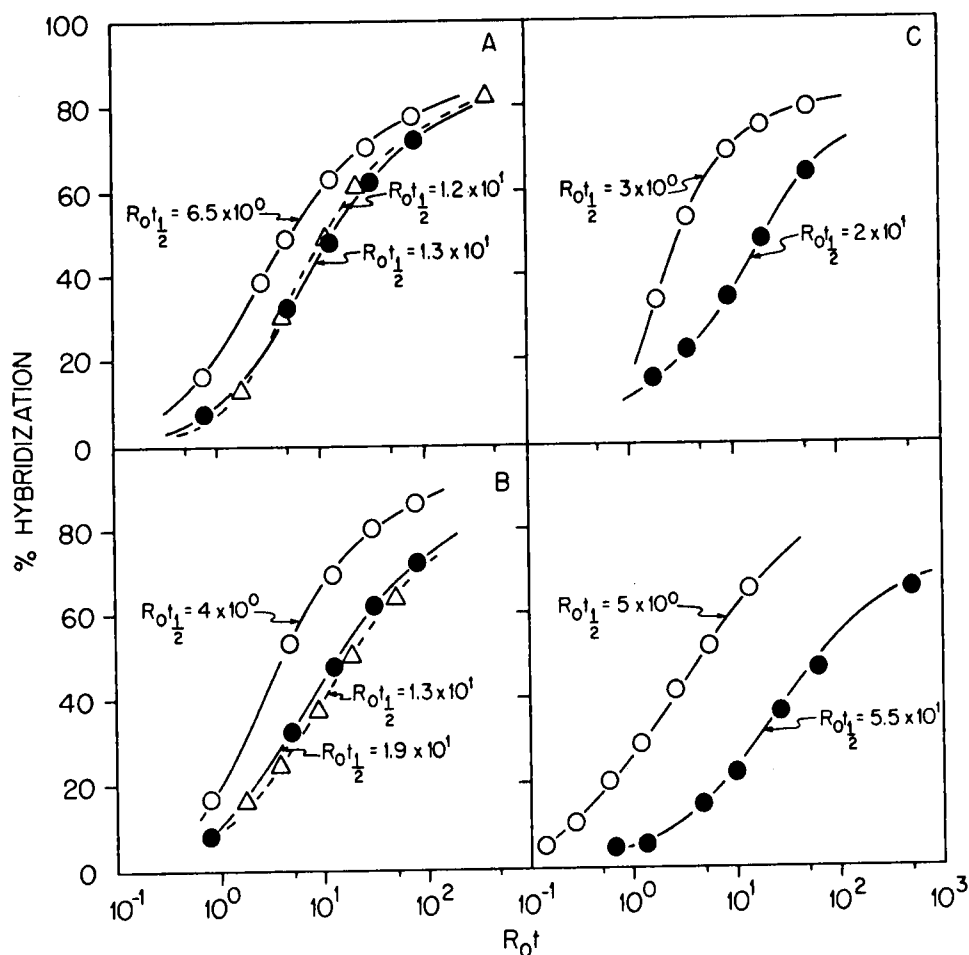


Figure V-5. The effects of hydrocortisone, dibutyryl cAMP and 8-bromo cAMP on albumin mRNA levels in hepa-2 cells.

RNA from cells treated with various compounds was hybridized to albumin cDNA. Panel A: RNA from cells treated with hydrocortisone, $\bigcirc$ — $\bigcirc$; tetrahydrocortisone ($10^{-6}M$), $\blacktriangle$ — $\blacktriangle$; and control, $\bullet$ — $\bullet$. Panel B: RNA from cells treated with Bt_2cAMP , $\bigcirc$ — $\bigcirc$; butyric acid ($10^{-3}M$), $\blacktriangle$ — $\blacktriangle$; and control, $\bullet$ — $\bullet$. Panel C: RNA from cells treated with 8-bromo cAMP, $\bigcirc$ — $\bigcirc$; control, $\bullet$ — $\bullet$. Panel D: RNA from cells treated with hydrocortisone and Bt_2cAMP , $\bigcirc$ — $\bigcirc$; control cells, $\bullet$ — $\bullet$. Each point represents the average of two determinations.

TABLE V-2

THE EFFECTS OF HYDROCORTISONE AND CYCLIC AMP ANALOGUES ON ALBUMIN
SYNTHESIS AND mRNA LEVELS IN HEPA-2 CELLS

Treatment	Total Synthesis		Relative Albumin Synthesis				Albumin	
	cpm/10 ⁶ cell/hr ($\times 10^{-4}$)	Expt.	Albumin/Total	Expt./	1/Rot _{1/2}	Control	(L·mol ⁻¹ ·sec ⁻¹)	Expt./
	Control		Control	Control	Control		Expt.	Control
Hydrocortisone	6.25	7.00	0.007	0.013	1.9	0.02	0.05	2.5
	4.94	6.02	0.016	0.041	2.6	0.09	0.21	2.3
Bt ₂ cAMP	4.25	5.23	0.016	0.046	2.9	0.09	0.26	2.9
	5.95	6.40	0.015	0.043	2.8	0.09	0.26	2.9
8-Bromo cAMP	3.59	5.79	0.020	0.096	4.8	0.10	0.36	3.6
Hydrocortisone plus Bt ₂ cAMP	3.48	4.69	0.008	0.091	10.5	0.02	0.22	11.1
	3.59	4.72	0.020	0.166	8.3	0.10	1.00	10.0
Tetrahydrocortisone	3.59	3.33	0.020	0.021	1.0	0.08	0.08	1.0
Butyric Acid	3.59	3.15	0.020	0.027	1.2	0.05	0.06	1.2

For each experiment, total protein synthesis, albumin synthesis and albumin mRNA levels were assayed in companion cell cultures treated identically throughout the course of the experiment. Total synthesis was determined on duplicate samples of cells labeled for 30 minutes. Relative albumin synthesis is the ratio of rates of albumin secretion from 60 to 180 minutes of labeling to rates of total protein synthesis. Rot_{1/2} values were determined from plots of 1/percent hybridization and 1/Rot and are expressed in reciprocal form to facilitate a direct comparison.

of these fluctuations, only actual data within each individual experiment are compared directly.

Table V-2 reveals that albumin mRNA, relative to control cell RNA, is increased approximately 2.4, 2.9 and 3.6-fold in hydrocortisone, Bt_2cAMP and 8-bromo cAMP-treated cells, respectively. Simultaneous exposure to hydrocortisone and Bt_2cAMP results in an approximately 10-fold increase in albumin mRNA. The yield of total cell RNA from treated cells was never more than 20% greater than the yield from untreated control cells ($1.8-1.9 \text{ mg}/10^8$ cells). Accordingly, the observed increases in albumin mRNA in total cell RNA must approximate increases in albumin mRNA per cell. A comparison of experimentally induced changes in albumin mRNA levels and changes in the relative rates of albumin synthesis (Table V-2) shows a correlation between the two parameters in all instances, regardless of treatment and regardless of basal levels of control cells. These data are graphically represented in Figure V-6.

From the data presented in Figure V-6, it may be seen that an approximate 1:1 relationship (slope = 0.88) exists between changes in albumin synthesis mediated by hydrocortisone and by cyclic AMP analogues and changes in albumin mRNA levels mediated by these factors.

IV. DISCUSSION

The continued synthesis and secretion of albumin by Hepa-2 cells is a biochemical phenotype which is characteristic of liver and has been maintained by these cells through neoplastic transformation of liver and adaptation of growth in tissue culture. The level of albumin

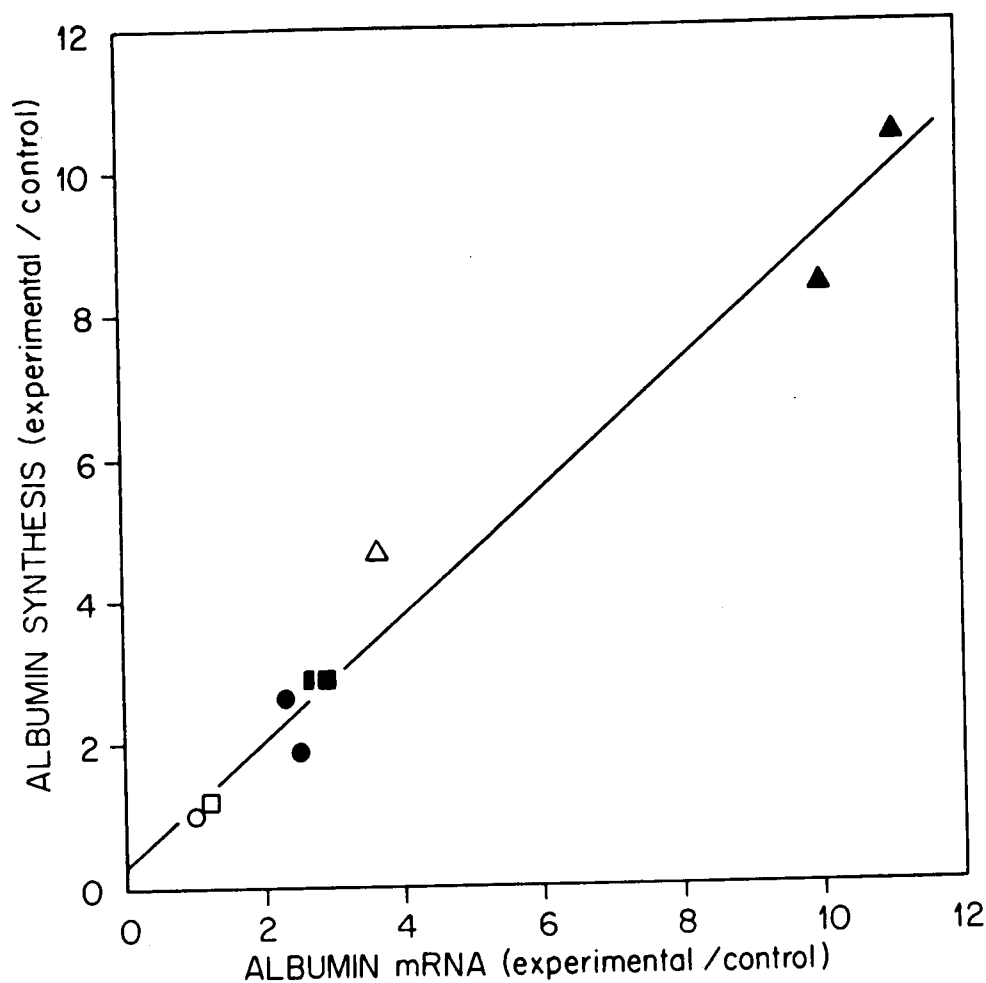


Figure V-6. The relationship between changes in albumin synthesis and mRNA levels in hydrocortisone and cyclic AMP treated cells.

From Table V-2, experimentally induced changes in albumin synthesis (experimental/control) are plotted as a function of corresponding changes in albumin mRNA sequence levels in cells treated with hydrocortisone, ●; tetrahydrocortisone, ○; Bt₂cAMP, ■; 8-bromo cAMP, △; butyric acid, □; hydrocortisone plus Bt₂cAMP, ▲. Least squares line is drawn in.

synthesis relative to total protein synthesis (these studies) and in absolute terms (9) are significantly reduced from levels associated with the intact liver. In addition, there appears to be extensive diversity in the levels of albumin synthesis in various clones of the Hepa parent cell line (9). Similar variation has been reported by Deschatrette and Weiss (31).

The ability of Hepa-2 cells to respond to hydrocortisone treatment by increasing albumin synthesis and mRNA levels shows that these cells have maintained a specific process which regulates the synthesis of albumin in the intact liver. In vivo (24, 82) and in isolated, perfused liver, glucocorticoids have been shown to stimulate the synthesis of serum albumin. In a sister clone, Hepa 1a, responsiveness to glucocorticoids seems to have been lost. Thus in addition to a diversity in the levels of albumin synthesis, regulatory variants are apparent in clones of the Hepa line. Loss of hydrocortisone responsiveness has been reported for clones of rat hepatoma cell lines as judged by the ineffectiveness of hydrocortisone in inducing tyrosine amino transferase (74). This loss of responsiveness seems to have been caused by a loss of cytoplasmic glucocorticoid receptor proteins.

The complexity of the hormonal regulation or modulation of albumin synthesis is indicated by the reports that synthesis is affected by growth hormone, thyroid hormone and by insulin (Chapter I). While the effects of the two former hormones have yet to be investigated in Hepa-2 cells, the addition of insulin to the growth media had little or no effect, possibly because insulin or insulin-like compounds are contained in the serum component of the growth media.

These studies have shown that the rate of albumin synthesis and secretion is stimulated in Hepa-2 cells approximately 3- and 4-fold by Bt_2cAMP and 8-bromo cAMP, respectively. Inductive effects of analogues of cyclic AMP on various liver enzymes has been described in the Introduction to the chapter. In addition to the induction of enzymes, cAMP has been implicated in the regulation of serum protein synthesis. The synthesis of fibrinogen, α_1 acid glycoprotein and acute phase globulins, which are serum proteins synthesized and secreted by the liver, are stimulated by the treatment of isolated, perfused liver with analogues of cAMP (82). In this context, the stimulation of albumin synthesis and mRNA by cAMP analogues seems more likely.

The finding that the 8-bromo derivative of cAMP was more active than the dibutyryl derivative is not surprising, since a similar observation has been made in the induction of TAT in hepatoma cells (150). It has been proposed that the biological activities of these compounds occur by different intracellular mechanisms in CHO cells (93). The dibutyryl derivative is thought to inhibit cAMP phosphodiesterase activity leading to higher intracellular cAMP; whereas 8-bromo cAMP stimulates cAMP-dependent protein kinases directly.

Maximum stimulation of albumin synthesis occurred during the simultaneous treatment of cells with hydrocortisone and Bt_2cAMP . This stimulation, approximately 10-fold, is a greater than additive effect; it is essentially a compounding of the individual effects of hydrocortisone and Bt_2cAMP on albumin synthesis. Synergistic effects of glucocorticoids and cAMP or cAMP-generating hormones such as glucagon and epinephrine have been shown for a number of specific enzymes such as

tyrosine amino transferase in liver cell suspensions (33), in cultured hepatoma cells (40) and in intact rats (153); glycerol phosphate dehydrogenase in rat brain cultures (14); and phosphoenol pyruvate carboxylase in cultured hepatoma cells (15). More will be said of this later.

Albumin mRNA

Quantitation of albumin mRNA by nucleic acid hybridization has provided unequivocal evidence that hydrocortisone and cAMP analogues mediate increases in albumin mRNA which are in direct proportion to the increases in rates of albumin synthesis.

The inductive effect of gonadal steroid hormones on specific mRNA in target tissues has been well documented by nucleic acid hybridization analysis for ovalbumin (78), conalbumin (71), vitellogenin (120), and α_2 _u-globulin (68). Using various mRNA translation systems for an indirect measure of mRNA levels, it has been possible to compare glucocorticoid-induced mRNA levels with rates of protein synthesis for tyrosine amino transferase (89, 109), glutamine synthetase (121), and tryptophan oxygenase (124). In all of these cases, induction of protein synthesis could be explained by elevated mRNA levels.

Regulation at the level of transcription has been the commonly accepted model of steroid action and might be invoked to explain glucocorticoid effects on albumin synthesis. However, as pointed out by Schimke et al. (123), there may be no single mechanism to explain hormone action. Teleologically, one might argue that in considering the evolution of regulatory processes, the importance of commonality of

mechanisms is secondary to the importance of the regulatory event itself. Alternate models of steroid action have been proposed (142). In one of the more interesting models, it has been proposed that glucocorticoids stabilize lysosomal membranes and in that way reduce the free intracellular concentration of lysosome-associated ribonuclease. Since lability of lysosomes is decreased in vivo and in isolated lysosomes by glucocorticoid treatment, this model seems to have some merit. It is difficult, however, to explain the specificity of the effects of glucocorticoids on the synthesis of certain proteins.

In a second model, sex steroids have been shown to increase the proportion of bound to free polysomes by altering the binding of polysomes to smooth endoplasmic reticulum (56). Related to this, it has been shown that hypophysectomy in rats decreases membrane-bound polysomes, albumin synthesizing polysomes and albumin mRNA (63). Since it is known that hypophysectomy eliminates circulating corticosteroids by removal of the source of adrenocorticotrophic hormone (ACTH), it is possible that these effects are a result of depletion of glucocorticoids (65). If the attachment of membrane-bound polysomes, of which albumin polysomes are a major component, is facilitated by steroid treatment, stabilization of albumin mRNA could result since Palmiter and Carey (96) have shown that accelerated degradation of ovalbumin mRNA and disappearance of oviduct polysomes occur upon acute withdrawal of circulating estrogen. Cause and effect relationships, however, are difficult to distinguish in these experiments.

As was mentioned in the Introduction to this chapter, recent experiments have shown that the induction of tyrosine aminotransferase

and phosphoenolpyruvate carboxykinase by cAMP could be explained by increased levels of mRNA coding for these enzymes. The data presented here show that a similar process may operate in increases in albumin synthesis mediated by cAMP. As to the exact nature of the process leading to increased levels of specific mRNAs, one may only speculate.

Published data indicate that glucocorticoids and cAMP, while they both mediate increases in specific mRNAs, act through different mechanisms. This statement is supported by a number of observations. The effects of cAMP on tyrosine aminotransferase and phosphoenolpyruvate carboxykinase mRNA levels are seen within 20 minutes, whereas glucocorticoid effects require hours. Both inductive effects, however, require ongoing protein and RNA synthesis (64, 154), and imply that the increases in specific mRNAs are the result of new synthesis. Furthermore, in the present studies and in other studies, glucocorticoids and cAMP interact in a compound manner, and not additively as one would predict if both substances were active at the same site. Thirdly, there is a wealth of evidence that shows that glucocorticoid effects are not mediated by changes in intracellular cAMP levels (142, 154). Finally, ample evidence exists to show that cytoplasmic receptors mediate glucocorticoid effects and that translocation to the nucleus and gene activation processes are time-dependent and explain the observed lag period between hormone administration and observable effect.

An important correlation in considering the mechanism of cAMP action as it effects specific protein synthesis may be that the phosphorylation of histone f_1 occurs in direct proportion to the level of induction of tyrosine aminotransferase by various analogues of

cAMP (150). The phosphorylation of nuclear proteins has been implicated in the regulation of gene transcription (66) and cAMP may act at this level. However, as Wicks (154) has pointed out, many cAMP mediated protein phosphorylation events occur which appear to have no biological function. Alternately, an increasing amount of interest is being directed towards post-transcriptional processing events which occur in the nucleus. Speculation abounds that the appearance of functional mRNA in the cytoplasm is a result of selective regulatory processes in the metabolism and transport of nuclear precursors of specific mRNAs. The majority of newly synthesized RNA is degraded in the nucleus and only a small portion, about 10%, reaches the cytoplasm (75). Nuclear processing involves many steps, including cleavage and ligation of large precursors representing primary gene transcripts, 5'capping, and the enzymatic addition of 3'polyadenylate sequences (14, 28, 75). Since the function of these processes is largely unknown, it is possible that one or more of these modifications enable specific mRNAs to "escape" from the nucleus and become functional in the protein synthesizing mechanisms in the cytoplasm. In theory, all of the processing steps are regulatable, possibly by the action of cAMP. It is not inconceivable that elevated levels of specific mRNAs could result from facilitated processing at any one of the steps mentioned.

Perhaps the most interesting observation from these and other studies involving the action of glucocorticoids and cAMP is that these compounds interact in a cooperative and apparently synergistic manner. This is perhaps best seen in the induction of tyrosine aminotransferase in liver cell suspensions (33), in rats (153) and in hepatoma cells (40),

and in the induction of glycerol phosphate dehydrogenase in rat brain cells (15). The induction of tyrosine aminotransferase in liver cells was absolutely dependent on the presence of hydrocortisone and glucagon or cAMP and none of the compounds was capable of inducing the enzyme when administered alone. In addition, the time of hydrocortisone treatments was the rate limiting step in enzyme induction. In the hepatoma cells, full induction of tyrosine aminotransferase by Bt_2cAMP required, as in the previous case, preinduction with glucocorticoid. While these experiments differ in the ability of each compound to induce synthesis of enzyme independently, the data indicate that the action of cAMP is to modulate the inductive event initiated by glucocorticoids. In the rat brain system, glycerol phosphate dehydrogenase induction by hydrocortisone was potentiated by cAMP and by agents which modify intracellular cAMP levels. As in the liver cell system, induction by cAMP alone was not observed. These results are consistent with a generalized model in which glucocorticoids, via a translocatable receptor protein, exert primary control of the transcription of a glucocorticoid "domain" of genes. Tomkins has defined the glucocorticoid domain as consisting of the battery of genes in an organism which are programmed to respond to glucocorticoid administration (145). On this background of glucocorticoid induced transcriptional activity, cAMP acts as a second level of control, amplifying or diminishing the initial transcriptional event, but initiating no new gene activity.

Modulation of Albumin Synthesis in Hepa-2 Cells

Following simultaneous treatment of Hepa-2 cells with hydrocortisone and Bt_2 CAMP, albumin synthesis is increased from approximately 1% to about 10% of total protein synthesis. In vivo, albumin synthesis comprises about 10% of total protein synthesis. Since albumin is synthesized in hepatocytes which comprise about 60% of total liver cell mass (84), relative albumin synthesis must approach 15% if all hepatocytes synthesize albumin equally. On the assumption that Hepa-2 cells are derived from hepatocytes, treatment with hydrocortisone and Bt_2 CAMP increase relative albumin synthesis to about 60-70% of in vivo levels.

Many explanations could be given for depressed albumin synthesis in tissue culture cells derived from hepatomas. It is not clear that all hepatocytes do in fact synthesize albumin equally (46) and it may be that Hepa-2 cells are derived from descendants of a low-producer in vivo. This could explain the clonal variation in lines derived from the parent cell line (9). In addition, it has been shown that neoplastic transformation can result in decreased albumin production in solid tumors (113). Again, however, it is possible that transformation has selected a type of liver cell that has low constitutive levels of albumin synthesis. Finally, it has been shown that albumin synthesis in primary cultures of chick liver decreases rapidly with time, implying that adaptation for in vitro growth may bring about changes in the synthesis of albumin (41). An interpretation here is that proper conditions have not been established for the maintenance of differentiated function.

Throughout the course of this dissertation, the term "induction" has not been used to describe the effects of hydrocortisone and cAMP analogues on albumin synthesis. The term "modulation" is preferred for reasons which are largely semantic. In the first place, these compounds raise an already ongoing process from relatively moderate levels (1% of total synthesis) to 10-fold higher levels. Treatment does not result in the appearance of new protein; merely more albumin. In this sense, perhaps tyrosine aminotransferase induction is the incorrect term, although it becomes difficult to assess basal levels in vivo and glucocorticoids contained in serum, a normal component of tissue culture media, complicate this issue in hepatoma cells lines. Secondly, maximal stimulation of albumin synthesis approaches but does not exceed in vivo levels of relative albumin synthesis. Hydrocortisone and cAMP analogues appear to have a restorative effect in Hepa-2 cells. In the third place, modulation of albumin synthesis by these compounds requires much longer than the classically "inductive" processes (97) and significant effects from either or in combination are only seen after 24-48 hours of treatment. Of interest, a similar 24 hour lag period in albumin modulation by hydrocortisone has been described in the treatment of a rat hepatoma cell line (138). In these studies, upwards of a 4-fold stimulation in albumin synthesis was detected after 48 hours of treatment. The growth of these cells was significantly slower than the growth of Hepa-2 cells, as evidenced by the 11-day doubling time of the former and the 18-hour doubling time for Hepa-2 cells. Additionally, the stimulatory effects of hydrocortisone treatment and the characteristic 24 hour lag period were observed in Hepa-2 cells arrested by serum

deprivation at mid-log phase and in growing cells treated not in mid-log phase ($10-15 \times 10^6$ cells/plate), but in late log/early stationary phase (35×10^6 cells/plate). The possibility, therefore, is unlikely that the stimulation of albumin synthesis by hydrocortisone treatment is somehow linked to the proliferate state of the cell. Studies of this nature were not performed with cAMP-induced albumin synthesis.

That the modulation of albumin synthesis by hydrocortisone and cAMP analogues require such a long period of treatment implies fundamental differences between this system and the classically inducible systems already described. Whereas the inducible systems appear to be direct effects of hydrocortisone and cAMP treatment, possibly the effects on albumin synthesis are secondary and indirect and result from a shifting biochemical emphasis or general state of differentiation resulting from treatment with these compounds.

An initial response of mammals to environmental stress such as starvation, injury, infection, and so forth, is increased secretion of glucocorticoids from the adrenal cortex into the circulatory system. Via the induction of enzymes involved in gluconeogenesis such as tyrosine aminotransferase, glucose is made available for elevated rates of glycolysis normally associated with stress situations. Without this response and because glycogen stores are limited, hypoglycemia and its potentially lethal effects could occur. It is more difficult to rationalize a physiological role for increased albumin synthesis under these same conditions since further amino acid depletion probably occurs. Since the osmotic properties of blood and corresponding blood volume are largely determined by serum protein, of which albumin comprises

about 50%, it may be that increased synthesis of albumin and other essential serum protein such as fibrinogen ensures adequate replacement of serum proteins with the increased likelihood of loss associated with the condition of environmental stress.

The maintenance of the differentiated state in tissue culture undoubtedly requires, in addition to the nutritional requirements satisfied by growth media, a specific intra- and extracellular balance of hormones, cyclic nucleotides, key divalent cations, as well as a multitude of growth factors. There is absolutely no reason to believe that the conditions required by one particular cell type are fulfilled by the inclusion of 10% fetal calf serum in the growth media used for the culture of Hepa-2 cells. Sato and his co-workers (47) have proposed that, given the right combination of factors, all cells may be grown in serum-free media and that these factors regulate the replications characteristic of the cell or tissue of origin. The studies described in this dissertation indicate that hydrocortisone and cAMP are two such factors and that the level of albumin synthesis in Hepa-2 cells may be elevated to levels approaching albumin synthesis in the hepatocyte in vivo. With the inclusion of other factors known to affect albumin synthesis, such as growth hormone and thyroid hormone, and the emerging technologies for the study of gene action, it may become possible to completely define the many events resulting in ultimate expression of the albumin gene.

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