

Clonal variation in albumin messenger RNA activity in hepatoma cells

(epigenetic control/geometric progression/wheat germ lysate)

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ABSTRACT The clonal variation in rate of albumin synthesis in hepatoma cells is described as a tool for the study of epigenetic control of differentiation. Previous studies have demonstrated that, from a population of hepatoma cells, variant subclones can be readily isolated that produce albumin at different rates. Each clonal variant had a characteristic rate of albumin production, and the clones clustered around discrete values that formed a geometric progression. The present experiments, using a cell-free protein-synthesizing system from wheat germ, show that albumin messenger RNA activity is directly proportional to the rate of albumin synthesis in three different hepatoma clones, thus suggesting a pretranslational control of albumin production. Possible hypotheses to explain the geometric pattern of clonal variation are discussed with respect to the organization and control of the transcriptional unit.

A possible tool for studying epigenetic control in mammalian cells is the clonal variation observed in cultured cells.† A prime example of this clonal variation is the variation in rate of albumin production in hepatoma cells in culture. Albumin is synthesized solely by the liver, although neither the structural gene nor the possibility of its expression are lost in cells of tissues that normally do not produce it. This has been clearly shown by the "activation" of the dormant albumin gene in a fibroblast by hybridization with a hepatoma cell (1). Variant clones that produce albumin at different rates can be readily isolated from any hepatoma cell population with a frequency that far exceeds that of gene mutation (2). Individual clones produce albumin at a constant rate during continuous cultivation; however, from each clone or subclone, new variants can be isolated (2). Individual clones that differ in rate of albumin production by more than ten-fold have very similar karyotypes (2). Other examples of phenotypic changes in cultured cells that occur more frequently than gene mutations are the loss of ability of myeloma cells to produce heavy chain immunoglobulin (3), and clonal variation in activities of tyrosine aminotransferase (4) and hypoxanthine-guanine phosphoribosyltransferase (5). Moreover, phenotypic changes occur at the same rates in cells of different ploidy, indicating that they may not be genetic alterations (6-9).

A possible key to an understanding of the nature of the epigenetic control of albumin production is the pattern of variation in its rate. In a study on clonal variation in rate of albumin production in hepatoma and hepatoma-fibroblast hybrid cells, individual clones were found to differ by as much as 200-fold (2). However, the variation was not random but discontinuous, for all hepatoma and hybrid clones clustered around values that

formed a geometric progression: a , $a(\sqrt{2})^1$, $a(\sqrt{2})^2$, $a(\sqrt{2})^3$, $a(\sqrt{2})^n$ ($a =$ a constant). The majority of clones fell into alternate classes in the series; these classes differed by a factor of 2. Because this pattern of variation is observed for many enzymes in hepatomas (2) and also in some normal tissues (2, 10), it appears that this phenomenon is widely distributed.

The geometric pattern of variation found with albumin production suggests that nucleic acids play a role in these phenotypic changes, because they are the only known molecules in the cell capable of serial duplications. To understand their possible role in this variation in gene expression, the cellular level at which the rate of albumin production is controlled must first be established. The present study was undertaken to distinguish between pretranslational and translational control by measuring the albumin messenger RNA (mRNA) activity in hepatoma clones that produce albumin at different rates.

MATERIALS AND METHODS

The origins and culture conditions of rat hepatoma cell line Fu5 and its subclones Fu5-5 and 967 have been described previously (1).

Two methods were used to extract RNA, *method A* for cytoplasmic RNA and *method B* for total cellular RNA. Both methods are slight modifications of techniques described by Palmiter (11). *Method A*. Cells harvested from roller bottles were suspended in 10 times their packed volume of buffer A (25 mM Tris-HCl at pH 7.5, 25 mM NaCl, 5 mM MgCl₂, 2% Triton X-100, 1 mg/ml of heparin) and homogenized in a Thomas tissue grinder with 15 strokes of a rotating Teflon pestle. The homogenate was centrifuged for 10 min at 27,000 × *g* to pellet the nuclei, the supernatant was decanted into another vessel, and an equal volume of buffer B (buffer A made 0.2 M MgCl₂ with 1 M MgCl₂) was added. After this was incubated for at least 1 hr at 0°, the precipitated ribonucleoprotein was pelleted through a pad of 0.2 M sucrose, 25 mM Tris (pH 7.5), 25 mM NaCl, and 100 mM MgCl₂. The pellet was resuspended in buffer A, to which an equal volume of 0.1 M NaOAc (pH 5.0) and 1/10 volume of 10% sodium dodecyl sulfate were added and RNA was extracted with phenol-chloroform as described elsewhere (12). The ethanol-precipitated RNA was then washed three times with 3 M NaOAc (pH 6.0) (11), and reprecipitated with ethanol and stored in this form at -20°. *Method B*. The entire crude cell homogenate, prepared as in method A, was made 0.05 M NaOAc (pH 5.0) and 0.1% sodium dodecyl sulfate; then total cellular RNA was extracted and salt washed as described above.

All solutions that came into contact with RNA were treated with diethyl pyrocarbonate to inactivate any ribonuclease activity (12). Twenty A₂₆₀ units of RNA were taken as equivalent to 1 mg of RNA.

Rat serum albumin released from the hepatoma clones into

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† For the purpose of this discussion I will define epigenetic control as that process that brings about a heritable change in cell phenotype but does not involve a change in the genetic potential of the cell (14-16).

Table 1. *In vitro* albumin mRNA activity in high (Fu5) and low (Fu5-5) albumin-producing hepatoma clones*

Hepatoma clone†	RNA extraction method	μg of albumin/ mg of protein per 72 hr	$\text{cpm} \times 10^{-3}$ in total protein	cpm in albumin	Percentage of total cpm in albumin
Experiment I					
Fu5 ₁	A	55.0 \pm 2.2	14.3 \pm 1.1	146 \pm 23	1.02
Fu5 ₂	A	55.0 \pm 2.2	16.2 \pm 2.3	180 \pm 9	1.11
Fu5-5 ₁	A	5.9 \pm 0.2	13.9 \pm 3.0	32 \pm 10	0.23
Fu5-5 ₂	B	5.9 \pm 0.2	16.5 \pm 2.2	18 \pm 1.2	0.11
Experiment II					
Fu5 ₃	B	55.0 \pm 2.2	9.6 \pm 1.0	157 \pm 7	1.63
Fu5-5 ₃	B	5.9 \pm 0.2	10.5 \pm 6.7	15 \pm 1.0	0.14

* Each *in vitro* reaction was performed in triplicate using 200 $\mu\text{g}/\text{ml}$ of polysomal RNA prepared from cytoplasmic ribonucleoprotein precipitated with Mg^{++} (method A) or from whole cells (method B) as described in *Materials and Methods*. Values for ^3H leucine incorporation (with SEM) into total protein and albumin are given for two independent experiments (I and II) using RNA extractions from three different preparations of each hepatoma clone. In experiment I, background incorporation of 40 cpm was subtracted from albumin, and 2100 cpm from total protein. In experiment II, the background cpm were 80 and 1580, respectively.

† Subscripts stand for different preparations of polysomal RNA.

the growth medium was assayed by microcomplement fixation (2) using antisera prepared from rabbits immunized with electrophoretically pure albumin as has been described previously (1).

In vitro translation of mRNA was carried out in a protein-synthesizing system prepared from commercial wheat germ (General Mills, Vallejo, Calif.), as described elsewhere (12). The complete reaction mixture in a final volume of 50 μl contained 20 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (pH 7.6) adjusted with 1 M KOH, 2 mM dithiothreitol, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 40 $\mu\text{g}/\text{ml}$ of creatine kinase, 100 mM KCl, 3 mM $\text{Mg}(\text{OAc})_2$, 100 $\mu\text{Ci}/\text{ml}$ of ^3H leucine (Schwarz/Mann; specific activity, 46 Ci/mmol), 19 unlabeled amino acids (25 μM each), and 10 μl of S30 (30,000 $\times g$ supernatant) wheat germ lysate (12). Reactions were performed at 25° and stopped by immersion in an ice-water bath. Incorporation of ^3H leucine into total protein was measured by precipitation with trichloroacetic acid and incorporation into albumin by immunoprecipitation with specific antiserum against rat serum albumin (12).

RESULTS

Albumin mRNA has been translated *in vitro* in lysates from liver (13), reticulocytes (14), and wheat germ (12). The wheat germ lysate system was chosen for the present study because of its low endogenous mRNA activity and ease of preparation.

The synthesis of rat serum albumin in the wheat germ lysate was measured by immunoprecipitation. The antiserum used in the assay was specific for rat serum albumin, since in immunodiffusion a single precipitation line formed against either rat serum or liver homogenate, and in both cases an arc with no spur developed when pure rat albumin was in an adjacent well. In addition, the radioactivity precipitated by the antiserum in the assay comigrated with pure rat albumin in sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis (12).

The kinetics of ^3H leucine incorporation into total protein and albumin in a wheat germ lysate stimulated with polysomal RNA from hepatoma cells (Fig. 1) were similar to those observed with liver polysomal RNA (12, 14). Incorporation increased linearly into both total protein and albumin for about 20 min and then leveled off. The percentage of total ^3H leucine incorporation in albumin varied between assays with polysomal RNA from a single hepatoma clone. Therefore, to compare albumin mRNA activity in different hepatoma clones, assays

were carried out with the same preparation of reaction mixture.

Three clones of hepatoma cells that produce albumin at different rates were chosen for assay: Fu5, 967, and Fu5-5. Because cells from different ampoules of the same lot of frozen cells will sometimes unexpectedly produce albumin at different rates (2), all experiments were done with cells from a single thawing. The three hepatoma clones produce, respectively, 55.0 \pm 2.2, 29.6 \pm 1.8, and 5.9 \pm 0.2 μg of albumin per mg of protein in 72 hr. Total polysomal RNA was extracted from the three hepatoma clones and assayed in the wheat germ lysate translating system. As can be seen in Table 1, where the highest-producing (Fu5) and lowest-producing (Fu5-5) clones are compared, there is clearly more ^3H leucine incorporation into albumin with RNA from the higher-producing clone. The RNA extracted from whole cells (method B) and from cytoplasmic ribonucleoprotein precipitated with Mg^{++} (method A) had similar albumin mRNA activities, indicating that most translatable albumin mRNA is cytoplasmic. The two hepatoma

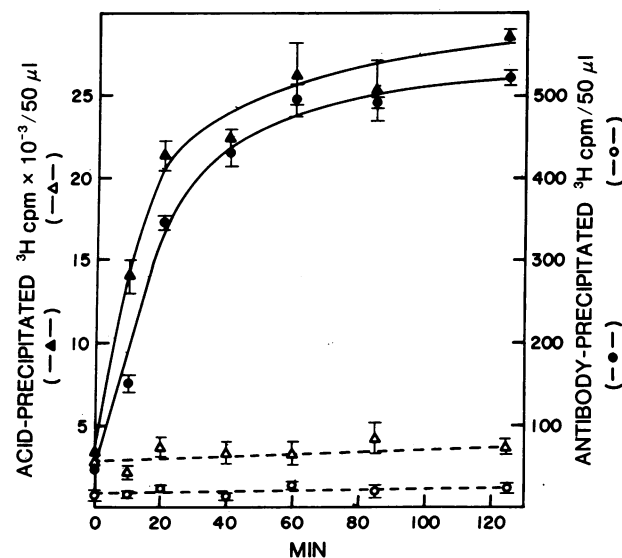


FIG. 1. Kinetics of ^3H leucine incorporation into total protein (triangles) and albumin (circles) in an *in vitro* protein-synthesizing system from wheat germ. Reaction mixtures of 750 μl were incubated at 25° with (closed symbols) and without (open symbols) 345 $\mu\text{g}/\text{ml}$ of polysomal RNA from cells of hepatoma clone Fu5 and duplicate 50 μl samples were taken at the times indicated.

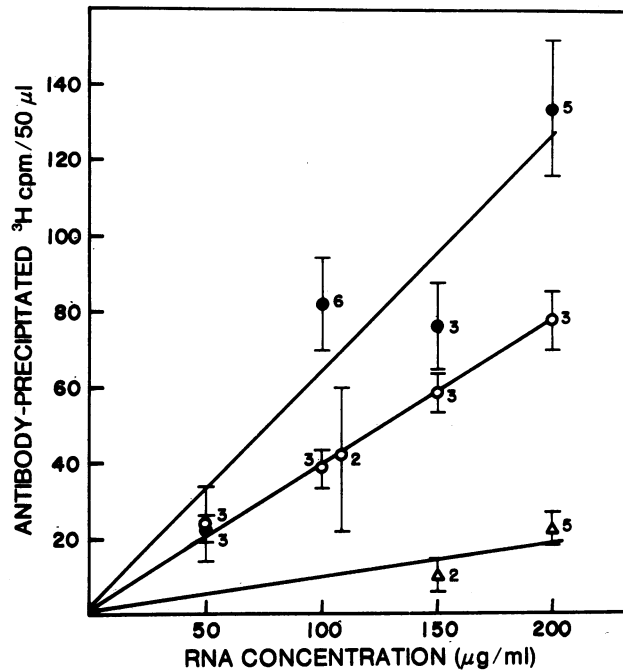


FIG. 2. Dependence of *in vitro* albumin synthesis in a wheat germ lysate on concentration of polysomal RNA from hepatoma clones that produce albumin at different rates. Hepatoma clones Fu5 (●), 967 (○), and Fu5-5 (△) produce, respectively, 55.0 ± 2.2 , 29.6 ± 1.8 , and 5.9 ± 0.2 μg of albumin per mg of protein in 72 hr. Each curve represents the best linear fit as determined by regression analysis of data from two independent experiments that were normalized to equal incorporation into total protein. The numerals next to each symbol indicate the number of determinations at that RNA concentration. Vertical lines represent SEM. A background incorporation of 15 cpm was subtracted in each experiment.

clones had similar total mRNA activity, but clone Fu5 had about eight to ten times more albumin mRNA activity than clone Fu5-5. Therefore, there is a direct correlation between the amount of albumin mRNA activity and rate of albumin production.

To assess more accurately the relative albumin mRNA activity in the three hepatoma clones, the dependence of *in vitro* albumin synthesis on RNA concentration for the three clones was compared in the same reaction mixture (Fig. 2). For all three clones there was a linear RNA dose dependence up to 200 $\mu\text{g}/\text{ml}$. Above this RNA concentration there was no further increase in [³H]leucine incorporation into total protein or albumin, possibly because of an inhibitory effect of ribosomal RNA, which makes up about 98% of polysomal RNA extracted.

When the slopes of the curves in Fig. 2 are plotted against the rate of albumin production for the three hepatoma clones, it is evident that cellular albumin mRNA activity is directly proportional to rate of albumin production (Fig. 3). Why the curve does not extrapolate to zero is unclear. However, considering the variation in the *in vitro* translating system, this difference from the ideal curve may not be significant. Similar relative values for the three hepatoma clones were obtained in three independent experiments.

DISCUSSION

The factors that establish and maintain the tissue-specific characteristics of a differentiated eukaryotic cell can be divided into two major categories: genetic and epigenetic (15-17). The genetic factors, which are encoded in the genome, represent the entire array of potentialities that can be expressed

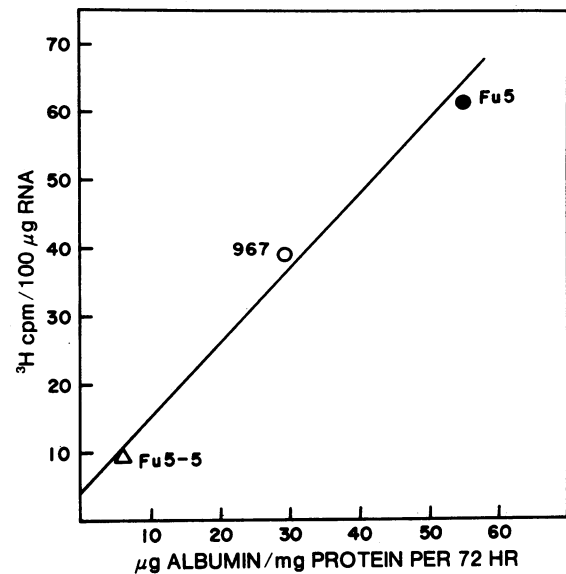


FIG. 3. Correlation between *in vitro* albumin mRNA activity (³H]leucine incorporation) and the rate of albumin synthesis in three hepatoma clones: Fu5, 967, and Fu5-5. The albumin mRNA activities for an RNA concentration of 100 $\mu\text{g}/\text{ml}$ are taken from the curves presented in Fig. 2.

throughout the life of the organism. A change in the genetic potential requires a gene mutation; that is, an alteration in the nucleotide sequence of DNA resulting in an altered gene product. The epigenetic factors would include all those factors that bring about a heritable change in cell phenotype without a change in the genetic potential of the cell. The predictable and sequential series of changes that occur in cell differentiation can be considered epigenetic because they usually do not involve loss or irreversible changes in the genome (17). Other examples of possible epigenetic changes include position-effect variegation in *Drosophila* and mice (18), phase changes in higher plants (19), alterations of serotype in *Paramecium* (20), phase variation in *Salmonella* (21), and transdetermination in the imaginal discs in *Drosophila* (22).

Although the molecular basis of these examples of epigenetic changes is unknown, they may have a common underlying mechanism because they share a number of characteristics: (a) they occur in somatic cells, except in the case of unicellular organisms; (b) they occur between or among predictable alternative states, either abruptly or via intermediate phenotypes; (c) they are reversible under certain physiological conditions; (d) their rate exceeds gene mutation rates; (e) once established, a given epigenetic phenotype tends to be constant, with continuous cell division in the absence of the environmental factor that incited it. Because these five characteristics also describe the clonal variation observed in cultured cells, an understanding of the molecular basis for the geometric pattern of variation in rate of albumin production may help to elucidate epigenetic control in general.

The results reported here establish that the rate of albumin production in three hepatoma clones is directly proportional to the intracellular levels of translatable albumin mRNA. Therefore, it would appear that alterations in the efficiency of translation are not responsible for the clonal variation in albumin production; rather the change(s) must occur in cellular processes that regulate specific mRNA levels, namely, mRNA transcription, processing, transport, and degradation.

At the level of transcription, the discontinuous shifts might reflect changes in the number of albumin structural genes.

There is evidence that some structural genes are moderately reiterated, for example, the genes for ribosomal RNA are reiterated a few thousand-fold (23); histones, 400-fold (24); and light-chain immunoglobulin, about 40-fold (25). The number of albumin genes per genome has not been determined, so it can be speculated that different rates of albumin production could result either from errors in replication, where the albumin gene would normally be reiterated to the same extent in all tissues, or from changes in the degree of tissue-specific gene amplification. Evidence in *Drosophila* suggests that the chromomere is the unit of both transcription and replication (26), and Callan (27) has proposed a model of the unit of replication, or replicon, in which irregular union at the base of a replication loop would result in tandem duplication. Successive tandem duplications would yield a geometric progression. Observations supporting tandem duplication in chromomeres are that there is a geometric relationship in DNA content of homologous chromomeres in salivary gland chromosomes of related subspecies of *Chironomus thummi* (28) and that the increase in DNA content of "DNA puffs" in *Sciara coprophila* occurs by stepwise doublings (29). If errors in replication occur in diploid cells, they may occur more frequently in cells in culture and in tumors that are rapidly proliferating.

The involvement of specific gene amplification in differentiation has been clearly shown only for ribosomal RNA in the developing oocyte (23). If gene amplification occurs in somatic cells it could be considered epigenetic by the criteria that I have discussed above. However, in three cases where it has been examined in somatic cells for the genes of ovalbumin (30), hemoglobin (31), and silk fibroin (32), there is only one gene per haploid genome in all tissues studied, although in the case of silk fibroin there are 10^6 copies of the gene per cell of the posterior silk gland due to polyploidization of the entire genome (33). If the clonal variation in albumin production were due to changes in specific amplification of the albumin gene, it would have to follow a geometric progression. One hypothesis for amplification of the ribosomal gene envisages the successive doubling of monocistronic circles (34–36). This would yield a geometric progression. However, evidence for a rolling circle intermediate in the amplification process does not support this hypothesis (37, 38).

If the albumin gene is not reiterated, a regulatory sequence that controls albumin production could be the site of changes in degree of reiteration. Recent evidence indicates that the sequence next to the hemoglobin structural gene is moderately repetitive (39), and 80–100% of mRNAs in HeLa cells are transcribed from sequences that are adjacent to repetitive sequences (40). Paigen *et al.* (41) have partially characterized a "regulatory gene" responsible for the tissue level of β -glucuronidase as being in close proximity, perhaps adjacent to the structural gene, but its nature is unknown. If such a regulatory sequence were repetitive and metastable, then changes in the number of reiterations could directly affect the rate of transcription, for example, by determining the number of polymerase-binding sites. Also, such a regulatory sequence could affect mRNA processing, transport, or stability by determining the size or stability of the initial transcript.

An alternative hypothesis that would not require a change in the number of structural or regulatory sequences could involve changes in the molecular organization of chromatin, where only part of a reiterated sequence is exposed for transcription. The geometric progression would then be a reflection of the molecular organization of the chromatin.

At the level of mRNA processing, there could be amplification of the mRNA by a regulated series of duplications by means

of a double-stranded intermediate, in a way analogous to replication of single-stranded (–) RNA viruses, such as vesicular stomatitis virus (42). Changes in the regulated number of duplications would result in a geometric increase or decrease in amount of mRNA.

The encouraging aspect of these hypotheses for the geometric variation in albumin production is that they can be tested experimentally by current techniques of nucleic acid hybridization, using a complementary DNA synthesized from albumin mRNA as a probe (43).

The further elucidation of the molecular basis of clonal variation may lead to a better understanding of the organization and control of the transcriptional unit, and in turn to the nature of epigenetic control of differentiation.

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