Immunofluorescence Analysis of the Time-Course of Extinction, Reexpression, and Activation of Albumin Production in Rat Hepatoma-Mouse Fibroblast Heterokaryons and Hybrids

MARYVONNE MÉVEL-NINIO and MARY C. WEISS
Centre de Génétique Moléculaire du Centre National de la Recherche Scientifique, 91190 Gif-sur-Yvette, France

ABSTRACT We have used a combination of a sensitive immunocytochemical stain for intracellular albumin, and Hoechst 33258 dye for identification of parental nuclei to investigate the time-course of extinction, reexpression, and activation of albumin production in fusion products of 1s (hyperdiploid) or 2s (hypertetradiploid) rat hepatoma cells with mouse fibroblasts (L cells or embryonic cells). In all combinations, the initial event is extinction of albumin production. Extinction occurs immediately after fusion when the mouse fibroblast is a normal embryonic (senescent?) cell. In the case of an L cell, rat albumin is synthesized and secreted during the first 12 h after fusion; no production of mouse albumin occurs. Thereafter, albumin production ceases. 8-12 d after fusion, young hybrid colonies are found to resume the synthesis of rat albumin (reexpression), and several days later the production of mouse albumin begins (activation). The patterns of reexpression and activation indicate (a) that chromosome loss is not necessary for either event to occur and (b) that the cells active in the synthesis of mouse albumin are a subpopulation of those cells already engaged in the production of rat albumin. We conclude that (a) extinction is mediated by diffusible factor(s) from the L-cell parent that act in the hepatoma nucleus to prevent the formation of new albumin messenger RNA; (b) reexpression and activation are gene dosage-dependent but extinction is not; and (c) previously active genes are more rapidly expressed than previously silent ones.

There are two reasons for studying the regulation of differentiation in somatic hybrid cells. First, the interactions that occur when differentiated cells are crossed with cells that do not express the same differentiation may reflect events that are controlled at the level of the gene: this is particularly clear for the phenomenon of activation and appears probable for that of extinction. Therefore, hybrid cells provide an experimental system amenable to molecular analysis of factors that directly regulate gene expression in mammalian cells. Secondly, there are arguments that the interactions that occur in hybrid cells reflect the very mechanisms involved in normal development and differentiation (see discussion in references 7, 17, and 19).

Much of the work that has been done up to the present has involved defining the experimental situations under which extinction (absence of expression), reexpression (reappearance of a previously extinguished function), and activation (new synthesis of a protein coded by a previously silent gene) occur. This phase of the analysis is now reasonably complete, and the results can be summarized as follows. When a cell that expresses a tissue-specific function is crossed with one that does not, this function is generally extinguished in the hybrid cells. The partner that causes extinction may be a cell of different histotype (12) or a nonexpressing variant of the same histotype (14). If the ploidy of the expressing parent is greater than that of the nonexpressing parent, extinction may not occur in the hybrid cells (18) and, in addition, expression of the homologous genes of the previously silent parent may be activated (31). Finally, loss of chromosomes from hybrid cells may lead to reexpression of an extinguished function (23, 42) and to activation of expression of the function by the genome of the silent parent (4). This ensemble of observations is most easily explained if we hypothesize that cells produce factors that (a)
inhibit the expression of genes foreign to their own phenotypic state, and (b) maintain the expression of genes specific to their phenotypic state.

Our aim in the present work has been to examine the time-course of extinction, reexpression, and activation, starting from the moment at which the parental cells are fused. This appeared to us a necessary first step in any analysis of the mechanisms underlying each of these phenomena. In the first place, knowledge of the rate at which these events occur should make it possible to eliminate some of the numerous hypotheses that could be advanced to explain them. Secondly, it is at present unknown whether cell divisions and/or changes at the chromatin level must precede the expression of a previously silent gene. Finally, changes in the relative gene dosage of the parental cells appear to result in diametrically opposed interactions, extinction on the one hand, or absence of extinction accompanied by activation on the other. We hoped that analysis of the time-course of these events in the appropriate crosses would clarify the relationship between extinction and activation.

To make the study feasible, we have developed a sensitive immunocytochemical staining procedure for albumin, allowing us to examine the expression of this liver-specific function in single cells. Albumin was chosen for study because it is rapidly secreted; the presence of intracellular albumin is, to first approximation, a direct reflection of its synthesis, so that difficulties of interpretation owing to turnover rates can be circumvented. Moreover, the availability of species-specific antisera makes it possible to monitor the activity of both parental genomes in interspecific fusion products.

Analysis of fusion products (heterokaryons, synkaryons, and early-proliferating hybrid cells) of a range of crosses between 1s or 2s albumin-producing rat hepatoma cells and diploid as well as subtetraploid mouse fibroblasts has revealed that extinction of albumin production is always the initial event after fusion. In the appropriate crosses, reexpression of rat albumin production, followed by the appearance of mouse albumin, occurs after five or more cell divisions.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The properties of all cell lines used in this work are summarized in Table I. Rat hepatoma clones 2sFou and Faö are descendants of clone H411EC3 (32) of the Reuber H35 hepatoma (34). 2sFou is a subclone of (2s) Faza 967-967cl.9 (27) and Faö is a subclone of Faza 967 (15), both selected for their resistance to 3 mM ouabain. Both lines are also resistant to 12 µg/ml 8-azaguanine and are deficient in hypoxanthine guanosine phosphoribosyl transferase (HGPRT); no revertants able to grow in HAT (1 x 10^{-8} M hypoxanthine, 4 x 10^{-7} M aminopterin, and 1.6 x 10^{-9} M thymidine; 26) selective medium have been observed. Clone BW1-1 (7) was derived from the mouse hepatoma line BW1 (37) and has no selective marker. c11D is a mouse fibroblast L-cell derivative (16) deficient in thymidine kinase (TK). EM is a strain of diploid fibroblastic cells isolated from embryos of the mouse strain DBA2 and provided by J. Jami (Institut de Recherche en Biologie Moléculaire, Paris, France). All cell lines were grown in modified Ham’s F12 medium (21) supplemented with 5% fetal calf serum. Cultures were maintained in Falcon plastic petri dishes in a humidified gas phase of 7% CO₂ and air. For selection of heterokaryons and hybrid cells, the medium was supplemented with 0.6 mM ouabain and HAT.

### Polyethylene Glycol-induced Cell Fusion

To analyze the first events after cell fusion, it was essential to obtain high yields of fused cells at densities that would permit us to distinguish unambiguously the fused cells from contiguous unfused cells. Two procedures were adopted; the first for study of events that occur 9 h or more after fusion, and the second for analysis of preparations 90 min to 12 h after fusion. In procedure 1, high-density monolayers (1.2 x 10⁴ cells/cm²) of parental cells mixed in equal numbers were treated with polyethylene glycol (PEG) 1000 mol wt (50% wt/wt in serum-free medium; 33) at 37°C for 45 s. The PEG was then aspirated, and the cells were rinsed three times with serum-free medium. After the last rinse the cultures were renewed with medium containing serum. 4-5 h later, the cultures were scored at a density of 5 x 10³ cells/cm² on 22-mm glass cover slips. This interval was necessary to permit recovery of the PEG-treated cells, and to allow coalescence of cells that had fused near the tips of elongated processes. Within 4 h, the reseded cells had attached and spread and could be processed for study. In procedure 2, mixed cultures of parental cells (ratio, 1:1) were inoculated at low density (2 x 10⁴ cells/cm²) onto cover slips. These cultures were treated with PEG as described above and could be processed very soon afterwards. There were two drawbacks to this procedure. First, satisfactory yields of heterokaryons could be obtained only when both parental cell types fused readily. Secondly, although the number of heterokaryons was relatively high during the first hours after fusion, this number dropped dramatically within 24 h, perhaps as a result of preferential formation of heterokaryons with small numbers of nuclei and their rapid conversion to synkaryons. The usual preparations of PEG (Koch-Light Laboratories, Colnbrook, Buckinghamshire, England; or Sigma Chemical Co., St. Louis, Mo.) produced enormous lethality; in our hands, only ultra-pure PEG from Merck (Darmstadt, Germany) resulted in acceptable levels of lethality (usually well below 20%). Selective medium was added 24 h after fusion. Zero time after fusion corresponds to the moment when the PEG rinses were terminated. The frequency of occurrence of hybrid colonies was about the same for crosses is (hyperdiploid) or 2s (hypertetradiploid) hepatoma cells with mouse L cells; 1-2 x 10⁶ of the cells present after PEG treatment. Hybrid colony formation was much less frequent when the mouse parent was a diploid mouse fibroblast (5 x 10⁷).

### Antisera

Rabbit antisera against pure rat and mouse serum albumin (RSA and MSA) were prepared and checked for specificity as described (27). These antisera are not species-specific but react with both albumins. They were used diluted 1:100 with phosphate-buffered saline (PBS). Species-specific antisera against rat or mouse albumin were obtained by immunoadsorption as described by Avrameas (2) and used at a dilution of 1:50 and 1:20, respectively. The species-specific antisera failed to react with the heterologous antigen when tested by either electroimmunodiffusion or immunofluorescence. Affinity-purified antitrat albumin antibodies were a gift of Dr. José Sala-Trepat (Laboratoire d’Enzymologie, Gif-sur-Yvette, France).

### Table I

Properties of Parental Cells

| Cell line | Species | Cell type          | Selective marker | Mean number of chromosomes (range) | µg secreted/10⁶ cells/24 h | % of fluorescent cells |
|-----------|---------|--------------------|------------------|-----------------------------------|---------------------------|------------------------|
| Faö       | Rat     | Hepatoma           | HGPRT⁺           | 52 (50-55)                        | 2.5                       | >98                    |
| 2sFou     | Rat     | Hepatoma           | HGPRT⁺           | 100 (96-102)                      | 5.8                       | >98                    |
| BW1-1     | Mouse   | Hepatoma           | None             | 64 (60-72)                        | 13                        | >98                    |
| c11D      | Mouse   | Fibroblast (L cell)| TK⁺             | 51 (46-54)                        | NT*                       | 0                      |
| EM        | Mouse   | Embryonic diploid fibroblast | None | 40 | NT* | 0 |

* NT, not tested.
Indirect Immunofluorescent Staining and Photomicroscopy

At various times, from 90 min to 4 wk after fusion, the cells on glass cover slips were rinsed with PBS and fixed in 3% formaldehyde for 1 min. The formaldehyde solution was then diluted 1:1 with methanol and aspirated, and pure methanol was added for 20 min at 4°C. This procedure fixed and permeabilized the cells without altering their morphology. Other fixation methods have been tried (acetic acid–methanol, I:100; 0.25% glutaraldehyde–ethanol) but did not give satisfactory results. In particular, the presence of acetic acid or glutaraldehyde in the fixative resulted in intense cytoplasmic fluorescence, and pure methanol or ethanol failed to preserve cell morphology. Antiserum (0.1 ml) against RSA or MSA was pipetted onto a paraffin sheet. Glass cover slips were placed cell-side-down on the paraffin and incubated for 30 min at 37°C in a humid chamber. The cover slips were washed twice with PBS, and then incubated for 1 h at room temperature with 1 ml of antibody against RSA or MSA. After two rinses the cells were stained with fluoresceochrome Hoechst 33258 (Serva, Heidelberg, Germany) according to Wright (43) and then rinsed with PBS. The cover slips were mounted with buffered glycerol on slides. Preparations were usually examined directly after fixation and staining but could be stored in the dark at 4°C for at least 1 wk without loss of fluorescence.

Preparations were examined under phase-contrast and epifluorescent illumination using a Zeiss UV microscope equipped with an HBO 200 W mercury lamp. A complete screening of the surface of the cover slip was carried out. For analysis of preparations from 7–28 after fusion, hybrid colonies were first located by use of a × 16 objective. Cells in the colonies were then screened for fluorescence with a × 40 oil-immersion objective. The combination of an interference excitation filter BP490, chromatic beam splitter FT 510, and barrier filter 520 nm located by use of a × 16 objective. Cells in the colonies were then screened for fluorescence with a × 40 oil-immersion objective. The combination of an interference excitation filter BP490, chromatic beam splitter FT 510, and barrier filter 520 nm was used for detection of fluorescein isothiocyanate (FITC) fluorescence. For 33258 Hoechst fluorescence, the excitation filter was UG1. Photographs were taken with a × 40 or × 100 oil-immersion objective, using Kodak Plus X Pan or Ilford HP5 films.

Measurements of Albumin Secretion

Albumin that had been secreted into culture medium was assayed by the electroimmunoassay method of Laurell (24), as detailed in Cassio et al. (6).

Specificity and Sensitivity of Immunofluorescent Staining for Albumin

Immunofluorescent staining of albumin in fixed mouse and rat hepatoma cells resulted in bright staining of the Golgi apparatus, where albumin is concentrated before secretion (30). The specificity of the reaction was verified by the use of affinity-purified antibodies; as shown in Fig. 1, this reagent produced a staining pattern identical to that observed with the appropriate dilutions of crude antisem. The cells to be used for crosses, Fao, 2sFou, and BW1-J (Table I), produce substantial amounts of albumin, and bright fluorescence was observed in at least 98% of the cells. The sensitivity of the method was evaluated by applying it to clones of rat hepatoma cells secreting 5–50-fold less albumin than Fao. These low albumin producers, Fso-5, FAl, and Fso-5-7, present an image different from that of Fao cells. Three classes of cells are observed: brightly and weakly fluorescent as well as negative (Table II). Most importantly, for cells of clone Fso-5-7 known to produce only 2% as much albumin as Fao, no brightly fluorescent cells are observed, although albumin is detectable in 50–60% of the cells. We thus estimate that the method would reveal the presence of albumin in cells producing only 3% of the Fao amount. Table II also shows that the same sensitivity is obtained whether adsorbed species-specific or nonsorbed antisera is used: for both, the rat and mouse (BW1-1) hepatoma lines, the fraction of cells in the various fluorescence classes is essentially the same whichever antisem is used.

Albumin Secretion Time for Hepatoma Cells

Various authors have assigned a secretion time of 0.5–1 h for mouse and rat hepatoma cells closely related to the clones used by us (3, 5). We have used two independent methods to verify that albumin is secreted very soon after its synthesis. First, immunoprecipitation of Fao culture medium has shown that radioactive albumin is present in the medium 60–90 min after the addition of [3H]leucine, and that the rate of accumulation of labeled albumin is a linear function of time thereafter (unpublished results from this laboratory). Secondly, Fao and 2sFou cells were treated with cycloheximide (Calbiochem-Behring Corp., Div. American Hoechst Corp., San Diego, Calif.), an inhibitor known to arrest protein synthesis. The rate of accumulation of "bleached" cells that no longer contain intracellular albumin was determined by immunofluorescence (Table III). That a large fraction of cells is reached within 60 min of treatment with effective doses of cycloheximide, and no cells remain fluorescent after 2 h, demonstrates that the intracellular pool of albumin is rapidly secreted, even in the absence of new protein synthesis.

Identification of Parental Nuclei

In preliminary experiments the parental cells were labeled with latex beads of different diameters (1.09–0.46 μm; Serva); heterokaryons showed the simultaneous presence of both types of beads. This procedure was subsequently abandoned, for two reasons. First, any lysis that occurred during PEG treatment resulted in beads being released into the medium; such free beads might then be taken up and cause mistakes in identification of cells. Secondly, this technique did not permit identification of the origin of each nucleus in heterokaryons containing three or more nuclei, a refinement that was essential for analysis of dosage effects. Staining of preparations with the fluorescent dye Hoechst 33258 (29) permitted unambiguous identification of mouse and rat nuclei: mouse nuclei are intensely stained and show numerous bright chromocenters, whereas rat nuclei show weaker, uniform staining. Moreover, with the use of appropriate filters, no interference occurs between FITC and Hoechst 33258 staining, so that it was possible on the same cells to identify both intracellular albumin and the origin of each nucleus.

In addition to permitting identification of parental nuclei in heterokaryons, the Hoechst stain also produced a characteristic staining pattern in synkaryons (isolated cells in which the parental nuclei have fused) and in proliferating hybrid cells. Very early synkaryons, observed 10 to 24 h after fusion, were often of irregular form, bilobular or multilobular, with bright chromocenters restricted to only part of the nucleus; such images must correspond to incompletely fused interphase nuclei. In oval or round synkaryons, the mouse chromocenters were first restricted to one half of the nucleus; after one or more divisions, the bright chromocenters became evenly distributed. Nuclei of proliferating hybrid cells were larger than those of the parent nucleus, and exhibited, like mouse nuclei, bright chromocenters. For the crosses analyzed in detail, nearly all cells of both parental types were killed in selective medium within 2 d, so that nuclei presenting bright chromocenters were thereafter restricted to heterokaryons and hybrid cells.

Evolution of Fusion Products from Different Crosses

The rate at which heterokaryons undergo nuclear fusion to give rise to synkaryons and proliferating hybrid cells is characteristic for each of the four rat x mouse combinations analyzed. The cross between mouse and rat hepatoma cells, BW1-J X Fao, resulted in the formation of heterokaryons that showed nuclear fusion primarily 48–72 h later; only few heterokaryons persisted by 6 d after fusion. In striking contrast, fusion of mouse L cells (c1D1) with either Is or 3s rat hepatoma cells (Fao or 2sFou) gave rise to heterokaryons that persisted a surprisingly short time: as early as 24 h after PEG treatment, over a third of the heterokaryons had already undergone nuclear fusion, and after only 48 h, few heterokaryons remained, and these were mostly cells containing large numbers of nuclei. The most striking results concern the cross of embryonic mouse fibroblasts (EM) with 2sFou: only a very small fraction of the heterokaryons gave rise to synkaryons, and proliferating hybrids were extremely rare. The heterokaryons persisted in appreciable numbers for several weeks, and in the few cases where nuclear fusion did occur, the mouse chromocenters, which stain intensely with Hoechst 33258, remained restricted to one pole of the nucleus.

Albumin Synthesis in Heterokaryons and Hybrids between Mouse and Rat Hepatoma Cells (BW1-J X Fao)

A number of hybrid clones between these two lines of albumin-producing hepatoma cells have been isolated and shown to coexpress the two parental albumins (6). This cross was chosen as a control not only to determine whether the fusion process itself could cause a transitory arrest of albumin synthesis but also to verify that fusion of cells of two different species does not interfere with the production of albumin. After immunofluorescent staining of preparations with species-specific antiserum against mouse or rat albumin, it was observed that albumin of both parental types continues to be synthesized at all times after fusion. Homokaryons and unfused cells were fluorescent as well. The fraction of negative heterokaryons or hybrid cells was <8%.

RESULTS

Early Events after Fusion: Extinction of Albumin Production

**FAO X CLID AND 2SFou X CLID:** Hybridization of Is rat hepatoma cells with mouse L cells is known to result in
FIGURE 1 The photomicrographs of this and the following micrographs (Figs. 4-6, and 9) are mounted to show: left, phase-contrast images; center, immunofluorescent staining of albumin, and right, appearance of nuclei after staining with Hoechst 33258. All photographs, unless otherwise specified, were made using a 100 x oil-immersion objective and the antiserum used to stain for albumin was rabbit anti-rat albumin, which reacts with both rat and mouse albumin. A, Albumin-producing BW1-J mouse hepatoma cells. The nuclei show bright chromocenters, which appear as intensely fluorescent spots. B, Fao rat hepatoma cells. The nuclei show the staining pattern characteristic of the rat: uniform pale staining with no bright chromocenters. C, Fao rat hepatoma cells for which albumin was visualized by using affinity-purified anti-rat albumin antibodies. The staining pattern is identical to that observed in A and B. In all cases, albumin is concentrated in the Golgi apparatus with little uniform fluorescence in the cytoplasm. Scale: 8.5 mm = 10 μm.

TABLE II

| Cell line (μg albumin secreted/10⁶ cells/24 h) | Fu5-5 (0.5) | FA11 (0.23) | Fu5-5 7 (0.05) | BW1-J (13) |
|---------------------------------------------|-------------|-------------|----------------|-------------|
| Adsorbed anti-RSA | Adsorbed anti-RSA | Adsorbed anti-RSA | Adsorbed anti-RSA | Adsorbed anti-MSA |
| % of cells* | RSA | RSA | RSA | RSA | RSA | RSA | MSA | MSA |
| ++ | 58 | 66 | 87 | 85 | 0 | 0 | 95 | 97 |
| + | 25 | 19 | 10 | 12 | 53 | 60 | 4 | 2 |
| - | 17 | 15 | 3 | 3 | 47 | 40 | 1 | 1 |

* +++ Brightly fluorescent; +, weakly fluorescent; -, negative.

These figures are based upon the analysis of at least 500 cells in each case. RSA and MSA refer, respectively, to rat and mouse serum albumin.

cells was undertaken to determine the kinetics and characteristics of extinction of albumin production.

A parallel study of the fusion products of 2s rat hepatoma cells with the same mouse cells was carried out to investigate the activation of mouse albumin synthesis. This experiment had its origins in the observation that hybrids produced by fusion of a 2s rat hepatoma cell with a 1s mouse cell of different histotype (fibroblast, lymphoblast) not only failed to show extinction of albumin synthesis but produced mouse as well as rat albumin (27, 31). We therefore anticipated that albumin production would not be extinguished in heterokaryons and hybrids from this cross, and intended to determine whether nuclear fusion invariably precedes the activation of mouse albumin production.

Very surprisingly, the production of albumin follows exactly the same pattern in fused cells from the two crosses (Figs. 2 and 3). Nearly all heterokaryons were positive at 6 and 12 h after fusion. Some images of "early" heterokaryons and hybrids are shown in Figs. 4 and 5. After 24 h when significant numbers of synkaryons as well as heterokaryons were present, the partial (10, 25, 39) or even total (38) extinction of albumin production. Analysis of heterokaryons and young hybrids resulting from the fusion of mouse L cells (cl1D) with 1s Fao cells was undertaken to determine the kinetics and characteristics of extinction of albumin production.
fraction of positive cells had dropped dramatically and remained low or negligible thereafter. Comparison of panels A and B of Figs. 2 and 3 shows that during the following 2 d the situation is not the same for heterokaryons as for synkaryons and young hybrids: among the former, a fraction of cells remained positive, whereas among synkaryons and hybrids positive cells were extremely rare. Panel C of Figs. 2 and 3 shows a regrouping of the heterokaryon population according to the ratios of parental nuclei. The cells that remained positive were those in which the ratio of rat nuclei was greater than mouse nuclei, showing that extinction is dependent upon gene dosage in heterokaryons. For all other classes of cells, extinction is observed between 12 and 24 h after fusion.

Because the secretion of albumin occurs very rapidly after its synthesis, the relatively slow rate at which extinction occurs is consistent with two possibilities. On the one hand, albumin synthesis could be arrested almost immediately after fusion, and the persistence of positive cells could reflect a defect in secretion of the protein that had been synthesized before fusion. On the other hand, both active synthesis and secretion could continue for at least 12 h, i.e., as long as positive cells are visible. To distinguish between these possibilities, preparations were treated with cycloheximide 4 h after fusion, to arrest protein synthesis. As shown in Table IV, within 2 h this treatment reduces to zero the population of positive cells, a time-course similar to that established for Fao and 2sFou cells (Table III). We conclude that the persistence of positive heterokaryons reflects the continued and active synthesis of albumin and that the positive cells secrete the protein at a normal rate.

**TABLE III**

**Effect of Cycloheximide on Intracellular Albumin of Fao and 2sFou Cells**

| Cycloheximide µg/ml | Time of treatment min | % of fluorescent cells* Fao | 2sFou |
|---------------------|-----------------------|----------------------------|-------|
| 0                   | 30                    | 99                         | 100   |
| 1                   | 30                    | 99                         | 100   |
|                     | 60                    | 70                         | 97    |
|                     | 120                   | 40                         | 3     |
| 5                   | 30                    | 100                        | 100   |
|                     | 60                    | 40                         | 78    |
|                     | 120                   | 3                          | 0     |
| 10                  | 30                    | 100                        | 99    |
|                     | 60                    | 13                         | 29    |
|                     | 120                   | 1                          | 0     |
| 50                  | 30                    | 99                         | 100   |
|                     | 60                    | 20                         | 25    |
|                     | 120                   | 0                          | 0     |

*These numbers are based upon the analysis of at least 300 cells for each sample analyzed. It has been shown previously that 30 µg/ml of cycloheximide reduces the incorporation of [3H]leucine by 96% in the closely related clone of rat hepatoma cells, Fu5-5 (42).
In light of these results, it appears probable that the albumin synthesized in heterokaryons reflects the continued utilization of a pool of messenger molecules contributed by the hepatoma parent. To verify that the cytoplasm of the heterokaryons provides an environment appropriate for the translation of albumin message, heterokaryons that had been “bleached” by treatment with cycloheximide were washed and returned to normal medium (see Fig. 6 for images). Within 6 h, the fraction of positive cells was essentially identical to that of untreated heterokaryons of the same age (Table IV). It can therefore be concluded that the cytoplasm of the heterokaryons does not contain factors that inhibit markedly the translation of albumin message.

Because albumin synthesis does continue for at least 12 h after fusion, the possibility had to be considered that a transitory activation of mouse albumin production occurs. Staining of preparations with adsorbed antiserum that reacts only with mouse albumin failed to reveal the presence of a single positive cell at any time during the first 3 d after fusion (more than 1,000 heterokaryons and hybrids examined for each cross).

From these results, it can be concluded that the arrest of albumin synthesis, which occurs between 12 and 24 h after fusion, is not the result of marked translation inhibition. Therefore, the simplest interpretation of our results is that the existing message population of the hepatoma parent is utilized, turned over, and not renewed.

2SFou X EM: The failure to detect activation of mouse albumin production in heterokaryons and the observation of extinction even for the cross 2SFou X c11D led us to examine a combination where the gene dosage in favor of the hepatoma parent is even further enhanced: 2s rat hepatoma cells X diploid mouse fibroblasts (Table I).

Analysis of albumin production in heterokaryons resulting from this cross gave highly unexpected results. Instead of occurring over an interval of 12-48 h, the extinction of albumin had already begun within 90 min after fusion, and was nearly total within 3.5 h. Fig. 6 shows an image of a negative heterokaryon at 90 min after fusion, and Fig. 7 the time-course of extinction: note the difference in time scale compared with Figs. 2 and 3. Fig. 7B shows that the effect of gene dosage in the different classes of heterokaryons is much less pronounced than in the preceding crosses: heterokaryons containing one mouse nucleus and up to four rat nuclei were usually negative. During the 3 wk that heterokaryons were present insignificant numbers, extinction remained total and activation of mouse albumin synthesis was never observed (at least 35 heterokaryons examined each day or two). Few heterokaryons underwent nuclear fusion. Only single synkaryons or up to two daughter hybrid cells were observed, and they very seldom gave rise to colonies.

The rapidity with which extinction occurs in this cross is not compatible with the interpretation offered for the crosses with
FIGURE 5 Very young hybrid cells. A, Fao and c11D cells were labeled with polystyrene beads (1.09 μm and 0.46 μm, respectively) before fusion. The hybrids observed 24 h after fusion show the two kinds of beads. They still contain intracellular albumin. Note that three hybrid cells are present in the field; the mouse chromocenters are localized primarily in one half of each nucleus. B and C, Hybrids between 2sFou and c11D 12 h after fusion. The two hybrid cells in B have presumably undergone one cell division and are nested close to two unfused Fao cells; albumin can be identified in all four cells. Hybrids in C have undergone two cell divisions. They still contain intracellular albumin. Note the presence of micronuclei and the characteristic images of hybrid nuclei with bright mouse chromocenters limited to only a portion of the nucleus.

TABLE IV

| Time after fusion | Number of cells | Number of cells |
|------------------|----------------|----------------|
|                   | 6 h            | 12 h           |
| Group             | +   | -  | %+ | +   | -  | %+ |
| Control           | 49  | 4  | 92 | 88  | 45 | 66 |
| Treated           | 0   | 52 | 0  | 0   | 48 | 0  |
| Treated and washed|    |    |    | 45  | 32 | 58 |

A series of cover slips was inoculated with 2sFou and c11D cells, and treated with PEG. 4 h later, they were renewed with medium containing 15 μg/ml of cycloheximide; cover slips were then processed 2 or 8 h later (corresponding, respectively, to 6 and 12 h after fusion). One series of cover slips was washed and renewed with the usual medium after 2 h of cycloheximide treatment, to permit the cells to recover and resume protein synthesis; they were processed 6 h later, corresponding to 12 h after fusion. In the group treated and washed, 20% out of 210 parental 2sFou cells were positive.

In this table, all classes of heterokaryons are grouped together; in the 12-h groups, the positive cells were more frequent in the classes where the ratio of rat nuclei was equal to or greater than that of mouse nuclei, as was already noted in Fig. 2B.

c11D cells. It is clear that embryonic fibroblasts provide an environment that causes either an inhibition of translation of existing albumin message or extremely rapid degradation of this population of molecules, or both.

Secondary Events after Fusion: Reexpression and Activation of Albumin Synthesis

Analysis of young hybrid cells and colonies formed by fusion of Fao or 2sFou with c11D cells revealed that the initial event of extinction may be only transitory (see also reference 14). Fig. 8 shows the percent of hybrid colonies containing cells that stain with species-specific antisera during the interval of 1–4 wk after fusion.

The reexpression of rat albumin production as well as the activation of mouse albumin synthesis was observed in two very different situations. In one case, cells of clearly hepatoma-like morphology were found to present very bright staining in a well-organized, triangular-shaped, Golgi-apparatus-like structure; such cells either were present as small nests within a colony of spread epithelial morphology, or they formed an entire colony (Fig. 9A and C; note the similarity to the images of Fao in Fig. 1). In the other case, colonies of spread epithelial morphology contained zones of cells that presented a diffuse...
FIGURE 6  A and B, Effect of cycloheximide on intracellular albumin in early 2sFou x c11D heterokaryons. A, Heterokaryon (two rat nuclei and one mouse nucleus) that was treated with cycloheximide 4 h after fusion and fixed 2 h later. The cell does not contain albumin. B, Heterokaryon (one nucleus from each parent) treated for 2 h with cycloheximide as in A, then rinsed and returned to normal medium. After 6 h of recovery (12 h after fusion) it exhibits intracellular albumin. C, Heterokaryon between 2sFou and normal diploid mouse fibroblasts, fixed and stained 90 min after fusion. There is no trace of intracellular albumin. Scale: 8.5 mm = 10 μm.

FIGURE 7  Time-course of extinction of albumin production in heterokaryons between 2sFou and normal diploid mouse fibroblasts. A, All classes of heterokaryons are grouped together. Various symbols (●, □, ▲) are used to represent the data from three independent experiments. B, Heterokaryons are grouped according to the ratios of parental nuclei: ○, rat = mouse; □, rat > mouse; ▲, rat < mouse. The insets show the total number of heterokaryons of each of the three classes. Note the difference in time scale compared with Figs. 2 and 3.

staining around the nucleus that could be confused at first with background fluorescence; with time, this staining became brighter and a Golgi-apparatus-like structure was visible in the form of a halo around the nucleus (Fig. 9 B). For the cross of Fa0 x c11D, reexpression was first observed at 9 d, and remained infrequent until 18 d. After 3 wk, 70% of
of extinction and activation presented here has revealed that contributions by the parental cells. Analysis of the time-course prevailing, depending upon the number of chromosome sets initiated functions in hybrid cells has been the apparent exclusion.

One of the more puzzling aspects of the behavior of differentiated functions in hybrids resulting from the cross of 2sFou with c11D (Fig. 8B). Both rat and mouse albumin were observed in hepatoma-like cells with a triangular Golgi apparatus, and in spread epithelial cells with a Golgi apparatus in the form of a halo. After 4 wk, a quarter of the colonies of spread epithelial morphology and the staining presented a halo image. The images observed for mouse and rat albumin staining were very similar. The percent of colonies containing positive cells when treated with non-cross-reacting species-specific anti-rat or anti-mouse albumin antiserum. Cells were scored as positive only when albumin was visible in a distinct Golgi-apparatus-like structure (see images in Fig. 9). In fact, the first indications of reexpression and activation, visible as a higher than usual background cytoplasmic fluorescence, were not scored. The data used to define each point were obtained by examining all of the cells present on a cover slip; each cover slip contained 30-50 colonies. Toward the end of the experiment, colonies were frequently touching one another so that determination of the exact number of colonies was sometimes difficult. Three morphological types of colonies were observed. Spread epithelial cells represented >50% of the colonies in the 2sFou × c11D cross and 80-90% in the Fao × c11D cross; hepatoma-like colonies represented 15% of the population in 2sFou × c11D cross and 5% in Fao × c11D cross; fibroblastic colonies that resembled the c11D parent rarely showed rat and never mouse albumin.

the colonies that contained positive cells were of spread epithelial morphology and the staining presented a halo image. Activation of mouse albumin production was observed later than reexpression, and was restricted to only one tenth of the colonies. However, toward the end of the experiment, ~50% of the colonies contained cells showing very diffuse fluorescence around the nucleus; the staining for mouse albumin remained weak, and such cells were not scored as positive because a clear Golgi-apparatus-like structure did not become apparent.

Reexpression and activation occurred more rapidly in hybrids resulting from the cross of 2sFou with c11D (Fig. 8B). Both rat and mouse albumin were observed in hepatoma-like cells with a triangular Golgi apparatus, and in spread epithelial cells with a Golgi apparatus in the form of a halo. After 4 wk, a quarter of the colonies of spread epithelial morphology became uniformly positive for albumin. The images observed for mouse and rat albumin staining were very similar. The main difference was that activation occurred later than reexpression (Fig. 8B). In addition, it affected a smaller number of cells: nests of cells showing intense staining for mouse albumin (Fig. 9C) were clearly smaller in size at comparable times than those active in the synthesis of rat albumin.

DISCUSSION
One of the more puzzling aspects of the behavior of differentiated functions in hybrid cells has been the apparent exclusiveness of extinction and activation (11, 18), one or the other prevailing, depending upon the number of chromosome sets contributed by the parental cells. Analysis of the time-course of extinction and activation presented here has revealed that these are not exclusive but sequential phenomena: in some combinations extinction is permanent, whereas in others it is only transitory, being followed first by reexpression, and then by activation. One example of transitory extinction is already known: hybrids between rat hepatoma cells and a dedifferentiated variant show extinction only during the first 2-3 wk after fusion (14). Study of young hybrids from other combinations may reveal that “absence of extinction” is in fact reexpression after transitory extinction.

The analysis of albumin production in heterokaryons and young hybrids derived by fusion of cells of different histotypic origins has confirmed previous reports that extinction occurs rapidly and does not require nuclear fusion (20, 40, 44). When 1s or 2s rat hepatoma cells are fused with mouse fibroblasts of a permanent line, the heterokaryons maintain active synthesis and secretion of rat albumin during the first 12 h after fusion; during this time there is no synthesis of mouse albumin. Then, extinction is observed, between 12 and 24 h after fusion. Let us now consider the types of mechanisms compatible with (a) the striking similarity in the pattern of early events in the crosses 1s and 2s rat hepatoma cells, and (b) the observed time-course of extinction.

Earlier observations on the gene dosage dependence of extinction in hybrid cells suggested that cells produce extinguishing factors in limited quantities, sufficient to be effective when confronted with another 1s genome, but not a 2s genome (11). The present work shows that this is not the case. Irrespective of the ploidy of the expressing parent, not only is extinction the end result, but the rate at which it occurs is the same. We conclude that the gene dosage dependence of extinction reflects not limited quantities of extinguishing factors but the course of later events that occur only after many cell divisions.

The rate at which extinction occurs is slower than would be expected if the arrest of albumin synthesis were the result of an inhibition at the level of translation. It was indeed possible to demonstrate that the heterokaryon cytoplasm provides an environment compatible with new synthesis of the protein. It is clear that the albumin messenger population contributed by the hepatoma parent is translated during the first 12 h after fusion. Thereafter, the arrest of albumin synthesis could be attributed either to a failure of this population to be renewed, or to depletion of some factor necessary for translation of the message. The former possibility seems the more likely one, because it has been demonstrated that extinction of a given function is correlated with the absence of the corresponding message in proliferating hybrid cells (9, 13, 25, 28). We thus conclude that the extinction of albumin production in the immediate fusion products of 1s or 2s rat hepatoma cells with L cells is the result of the action of diffusible factor(s), provided by the L cell parent, that act(s) in the hepatoma nucleus to prevent the synthesis of new albumin mRNA.

This interpretation does not apply to the situation encountered in the cross of 2s rat hepatoma cells with embryonic mouse fibroblasts. Here, the arrest of albumin synthesis is nearly instantaneous. Only 90 min after fusion, a significant fraction of heterokaryons no longer contains intracellular albumin, even though newly synthesized albumin is not secreted for nearly an hour. The albumin message population contributed by the hepatoma parent is either not translated or is subject to rapid degradation. It is perhaps relevant to note that mouse fibroblasts undergo relatively few divisions before en-
tering the state referred to as “senescent,” and we suggest that the situation we have encountered reflects a previously unsuspected feature of senescent cells: their inability to conserve and/or to utilize a pool of exogenous mRNA.

Our observations of the patterns of reexpression and activation in young hybrid colonies of Fao or 2sFou × c11D revealed two striking results: (a) two very distinct classes of cells became positive for albumin and (b) both reexpression and activation occurred in cells of the two types. A first class consists of cells that present a typical hepatoma-like morphology, with intracellular albumin concentrated in a Golgi-apparatus-like structure and few mouse chromocenters in the nucleus. Such cells have probably lost mouse chromosomes. In the second class of cells, albumin was concentrated as a halo around the nucleus. Albumin staining in such cells was very weak initially, and became more intense with time. The morphology of these cells is flat epithelial and albumin is not concentrated in a typical Golgi-apparatus-like structure. Such hybrid cells have probably not undergone chromosome segregation, because they constitute the majority of all hybrids (>50%). It seems likely that the bulk of this hybrid population slowly reaches an equilibrium state that permits the expression of albumin. These observations strongly suggest that the same mechanisms are involved in both reexpression and activation, and furthermore that loss of chromosomes of the fibroblast parent is not the unique mechanism leading to reexpression and activation. That reexpression precedes activation might reflect a requirement for changes at the chromatin level before a heretofore silent gene can be expressed (35, 41).

The results of these studies render understandable the failure of several investigators to obtain activation in heterokaryons (1, 8, 36); many days and cell divisions precede activation of mouse albumin production. If the results we have obtained apply also to other systems, it will be difficult to use heterokaryons or injection systems to identify the substances involved in reactivation of a silent gene. A more positive prospect is that changes at the chromatin level may be directly involved in activation, and some aspects of this problem are now directly amenable to experimental study. By contrast, the time-course with which extinction occurs is favorable for an attempt to identify the factors involved. Indeed, the techniques developed here have also been used to demonstrate that a transitory extinction of albumin production occurs when L cell cytoplasts are fused with rat hepatoma cells, following an initial time-course similar to that described above (22). We anticipate that it should soon be possible to identify the nature of the molecule(s) responsible for extinction, to determine their tissue distribution, and perhaps to elucidate the site of action.

Figure 9 Reexpression and activation of albumin production in hybrid colonies, detected by using species-specific antisera. A, Hybrid colony of Fao × c11D, 16 d after fusion. Reexpression of rat albumin production is seen in a nest of cells in the colony; note the granular appearance of the cytoplasm of these cells. (40 x oil-immersion objective). B, Reexpression in a hybrid colony of 2sFou × c11D, 16 d after fusion. Albumin is not concentrated in a triangular Golgi-apparatus-like structure but is localized around the nucleus as a halo (100 x oil-immersion objective). C, Activation of mouse albumin production in a hybrid colony of 2sFou × c11D, 15 d after fusion. Note the similarity in the image of rat albumin in A (reexpression) and mouse albumin in C (activation) (40 x oil-immersion objective). Scale: A and C, 10 mm = 30 μm; B, 8.5 mm = 10 μm.

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