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Repression of Quiescence-Specific Polypeptides in Chicken Heart Mesenchymal Cells Transformed by Rous Sarcoma Virus

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Chicken heart mesenchymal cells do not proliferate in medium of physiological composition containing plasma (S. Balk, Proc. Natl. Acad. Sci. USA 77:6606–6610, 1980). To understand the molecular events involved in cell quiescence and in the initiation of cell division under physiological conditions, we examined the differences in the patterns of protein synthesis of quiescent, hormone-stimulated, and Rous sarcoma virus-transformed chicken heart mesenchymal cells. We describe the expression of a 20,000-kilodalton (kDa) polypeptide actively synthesized by quiescent cells but not by their transformed counterparts. Normal chicken heart mesenchymal cells stimulated with epidermal growth factor and insulin also repressed the synthesis of the 20,000-kDa polypeptide while actively growing but synthesized increasing amounts of the protein at high cell density (confluence). The synthesis of the 20,000-kDa protein is not restricted to chicken heart mesenchymal cells, since confluent, density-arrested chicken embryo fibroblasts also expressed high levels of the protein. Transformed chicken heart mesenchymal cells and embryo fibroblasts did not synthesize the protein even at high cell density. The 20,000-kDa polypeptide accumulated in the culture medium.

It is argued that oncogene products result in abnormal cell proliferation; yet there are few systems in which division of transformed cells continues while their normal counterparts are quiescent. One such system has been described by Balk and co-workers in which chicken heart mesenchymal (CHM) cells do not proliferate in medium containing heat-inactivated, defibrogenated plasma, whereas transformed or hormone-stimulated cells undergo several rounds of division (3, 6). Although certain components present in serum, such as growth factors released during platelet breakdown, are absent in plasma, cell proliferation under these more physiological conditions is very active for other cell types such as chicken embryo fibroblasts (CEF; 3). Thus, in this system, quiescence does not depend upon growth factor depletion or density arrest but is determined by nonresponsiveness of the CHM cells to endogenous growth factors present in plasma (5). Because the reentry of quiescent CHM cells into the cell cycle can be triggered by the addition of a variety of hormones and growth factors or by oncogene products such as $pp60^{v\text{-}src}$, the CHM cells provide a favorable system to investigate the initiation of cell division in normal and transformed cells. In addition, the use of CHM cells is favorable to the investigation of cell quiescence under physiological conditions. Many investigators have argued that tissue cells grown in culture contain an element of artifact, since in the adult body most cells are embedded in a fixed matrix and do not divide (25). It should be noted that cells in vivo are normally not exposed to growth factors present in serum used for cell culture. In the instances of tissue replacement and wound healing, growth is tightly controlled and does not lead to overgrowth of the tissue. Therefore, proper mechanisms determining the initiation and cessation of cell division (quiescence) must be provided to preserve the integrity of the tissue. Recently, much effort has been devoted to the understanding of feedback mechanisms involved in growth control (28, 36) and to the characterization of negative modulators of cell division (33).

We used CHM cells to investigate the initiation of cell proliferation in a system characterized by quiescence under physiological conditions. In this report we describe changes in protein biosynthesis that occurred after hormone stimulation or Rous sarcoma virus (RSV) transformation of CHM cells. We observed that few gene products were newly synthesized by cells reentering the cell cycle after hormone stimulation or transformation. In contrast, several abundant proteins synthesized by normal cells were down regulated after transformation and the initiation of cell division. We characterized more thoroughly the synthesis of polypeptides with an M_r of 20,000 expressed by quiescent CHM cells but repressed in their transformed counterparts. Synthesis of these proteins was also repressed upon hormone stimulation of CHM cells but became detectable when the stimulated cells became confluent. We compared the expression of the 20,000-kilodalton (kDa) polypeptides in normally dividing and transformed cells and analyzed the synthesis of the same polypeptides in density-arrested CEF.

MATERIALS AND METHODS

Cell isolation. The CHM cells were isolated from the ventricles of 8- to 12-week old cockerels (SPAFAS, Inc., Norwich, Conn.), as described by Balk (3). The ventricles were minced and subjected to 8 or 10 cycles of trypsinization. The cells from the last cycles were pelleted by centrifugation, washed in a physiological electrolyte-glucose solution, and suspended in complete SC-12 medium (4). Several tissue culture dishes were seeded with the cell suspension. After an incubation period of 3 h, the CHM cells had attached to the surface of the dish and could be isolated from myocytes, erythrocytes, and cell debris by extensive washes in physiological electrolyte-glucose solution. The CHM cells were then cultured in SC-12 medium containing 5% heat-inactivated, defibrinogenated rooster plasma, epidermal

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growth factor (EGF) at 100 ng/ml, and insulin at 10,000 ng/ml (4, 5). Confluent CHM cells were transferred to medium containing 10% plasma and subcultured into 35-mm dishes for experiments. The experiments described in this report were performed on secondary cultures of CHM cells. While the SC-12 medium allows for optimal growth of chicken cells, all results were reproduced with cells cultured in Dulbecco modified Eagle medium (DMEM) containing plasma.

Early passages of CEF were grown in DMEM containing 10% serum and subcultured in 35-mm dishes in plasma- or serum-containing medium. Results obtained with CEF were reproduced with SC-12 medium. Cultures of CHM cells or CEF were infected with the Schmidt-Ruppin A strain of RSV or with RSV NY 72-4, a temperature-sensitive mutant provided by H. Hanafusa (24). EGF was purchased from Biomedical Technologies, Inc., Cambridge, Mass., and insulin was obtained from Sigma Chemical Co., St. Louis, Mo.

Labeling conditions and polyacrylamide gel electrophoresis. In analyses of patterns of protein synthesis, CHM cells and CEF seeded in 35-mm dishes were metabolically labeled with 50 to 250 μCi of L-[^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) per ml for 1 to 2 h. After the labeling, the culture medium was collected, centrifuged to eliminate detached cells, and stored frozen at −70°C. The cells were washed extensively in ice-cold physiological electrolyte-glucose solution, scraped off the dish, and collected by centrifugation. Cell pellets were lysed in O'Farrell lysis buffer (23) containing 0.2% sodium dodecyl sulfate (SDS). Complete cell lysis was achieved by vortexing and by freezing and thawing the cell lysate once or twice. Cell counts from parallel cultures were determined with a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) For analysis on one-dimensional SDS-polyacrylamide gels, the proteins were acetone precipitated from cell lysates or culture media and dissolved in an SDS-containing buffer. The incorporation of L-[^{35}S]methionine was determined by hot trichloroacetic acid precipitation.

One-dimensional SDS gel electrophoresis was done by the method of Laemmli (21) on slab gels consisting of a 10 to 14% exponential gradient of acrylamide. Two-dimensional gel electrophoresis was done by the method of O'Farrell (23) with the conditions described by Bédard and Brandhorst (7). Under these conditions, proteins having an isoelectric point between 5 and 7 and a molecular weight between 10,000 and 230,000 can be analyzed. Molecular weights were determined from the migration of known standard proteins, and the pH range of the isoelectric-focusing gels was measured with a pH meter after elution of the ampholines. Detection of radioactively labeled proteins was accomplished by autoradiography or fluorography of dried gels on X-AR films (Eastman Kodak Co., Rochester, N.Y.). Sodium salicylate was used for fluorography (10). For two-dimensional gels, the films were developed after exposure to 10^{10} disintegrations of acid-precipitable radioactive proteins loaded onto the isoelectric-focusing gel. Similarly, equal numbers of counts of radioactive proteins were loaded on each lane of one-dimensional SDS gels.

Antiserum preparation. The major isoform of the quiescence-specific p20 polypeptide was isolated from the culture medium of dense quiescent CHM cells maintained in plasma-free medium. Proteins were recovered from the medium by acetone precipitation, dissolved in O'Farrell lysis buffer containing 0.2% SDS, and separated by two-dimensional gel electrophoresis. The major form of p20 was located by Coomassie blue staining and eluted in a buffer consisting of 50 mM ammonium bicarbonate (pH 8.5) and 0.1% SDS at 37°C from excised gel pieces. The eluted protein was suspended in phosphate-buffered saline (PBS) and injected in the popliteal lymph nodes of two rabbits by the methods of Siegel et al. (31) and Resh and Erikson (27). The immunization resulted in a high-titer serum against the p20 protein in one of the rabbits; we designated the antiserum 601. Using known amounts of isolated p20, we determined that 100 ng or 5 pmol of protein could be precipitated by 1 μl of serum. Two-dimensional gel analysis of radiolabeled proteins and Western blotting analysis of total proteins indicated that the major 20-kDa polypeptide was the only protein recognized by the serum. The minor 20-kDa isoform was not recognized even though it appeared to be related structurally to the major isoform when analyzed by limited digestion with the protease V8 (data not shown).

Immunoprecipitations and Western blotting. Samples of cell lysate or culture medium containing radiolabeled proteins were suspended in RIPA buffer (8) and immunoprecipitated on ice with the 601 antiserum under conditions of antibody excess. The immunoprecipitate complex was isolated by binding to *Staphylococcus aureus*, and the 20-kDa polypeptide was analyzed by polyacrylamide gel electrophoresis and fluorography. For Western blotting, we followed the protocol of Towbin et al. (32) to electrophoretically transfer proteins from SDS-polyacrylamide gels onto nitrocellulose sheets. We used a commercially available biotinylated, affinity-purified anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, Calif.) to visualize the immunocomplex. In this system a standard peroxidase staining protocol using 4-chloro-naphthol and imidazole (14) as substrates is followed to reveal the final immunocomplex.

Indirect immunofluorescence microscopy. CHM cells were grown on glass cover slips and transferred to plasma-containing medium to attain quiescence. The cells were fixed in 3.7% formaldehyde in PBS for 15 min at room temperature. After several washes, a fraction of the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and then rinsed extensively in PBS. The reaction with preimmune or 601 antiserum (diluted 100-fold) was carried out for 1 h at room temperature in the presence of 10% calf serum. After several washes, a fluorescein-conjugated goat anti-rabbit immunoglobulin G (Miles-Yeda, Naperville, Ill.) was added and incubated in the dark under conditions described above. The cells were washed extensively and mounted in 90% glycerol-PBS containing 0.1% *p*-phenylenediamine, an antibleaching agent. A Zeiss microscope equipped with epifluorescent illumination was used to observe the immunofluorescence patterns, which were photographed on Kodak TRI-X films by using an automatic exposure meter.

RESULTS

We examined the patterns of protein synthesis in normal quiescent, normal hormone-stimulated, and RSV-transformed CHM cells. Secondary cultures were metabolically labeled with L-[^{35}S]methionine in SC-12 medium containing 10% plasma with or without EGF and insulin. RSV-transformed cells were infected 4 days before labeling and were grown without EGF and insulin like their uninfected quiescent counterparts. The hormone-stimulated CHM cells were cultured for 3 days in the presence of 100 ng of EGF per ml and 10,000 ng of insulin per ml before being labeled. This combination of EGF and insulin results in a division rate approximating that of RSV-transformed cells (6). The radioactively labeled proteins were analyzed by two-dimensional

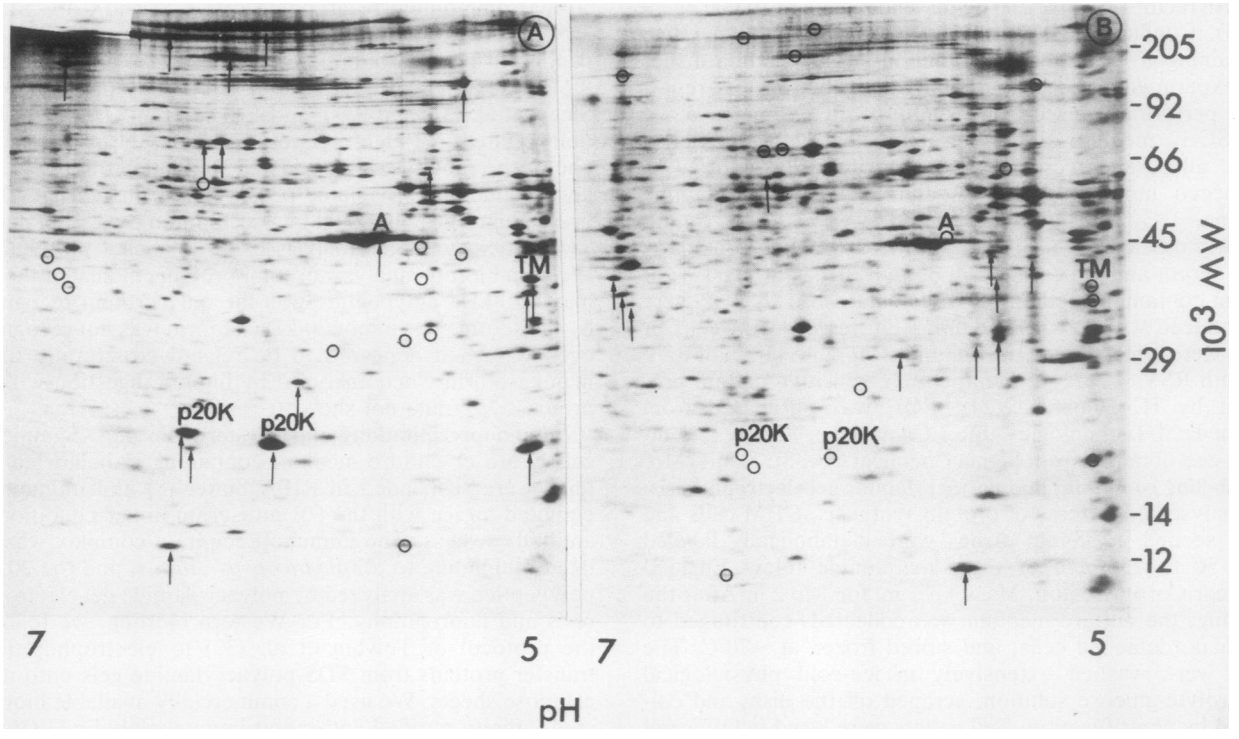

FIG. 1. Two-dimensional gel analysis of proteins synthesized by normal quiescent and transformed CHM cells. Proteins were metabolically labeled with L-[^{35}S]methionine for 2 h and analyzed by two-dimensional gel electrophoresis and autoradiography. (A) Pattern for normal quiescent cells; (B) pattern for RSV-transformed cells. Arrows point to polypeptides more intensively labeled in one pattern, and circles indicate the corresponding areas on the alternate gel. Polypeptides identified by symbols are described in the text.

gel electrophoresis (23) followed by autoradiography. The patterns of protein synthesis of quiescent and transformed CHM cells are shown in Fig. 1A and B, respectively. The polypeptides uniquely represented or more intensively labeled in one pattern are indicated by arrows, and the corresponding areas on the alternate gel are indicated by circles. Only a few percent of the several hundred polypeptides analyzed showed detectable differences in labeling intensity. Most of the transformation-enriched polypeptides (arrows in Fig. 1B) were weakly labeled and probably corresponded to low-abundance gene products. This observation is in agreement with the results of a previous investigation of RSV-transformed cells (26). These polypeptides appeared to be transformation specific, since they were not detected in the pattern for hormone-stimulated cells (data not shown). Many of the quiescence-enriched proteins missing in transformed cells (arrows in Fig. 1A) were actively synthesized in normal cells. By mass, they constituted a group of abundant proteins, and they were easily detectable by staining with Coomassie blue (data not shown). In some instances, they occupied the positions of known proteins on two-dimensional gels. Polypeptide A in Fig. 1 has a molecular size of 44 kDa and an isoelectric point of about 5.5, characteristic of actins. Polypeptides TM migrated in a region of the gel occupied by tropomyosins (molecular size of 36 kDa and pI of 5). The high-molecular-weight complexes showing streaking in the first dimension (molecular size of 190 to 230 kDa) were also present in the culture medium and may be associated with the cell surface. The identification of the polypeptides described above remains tentative but is in agreement with the results reported by several investigators. The decreased synthesis in transformed cells of cell surface proteins such as fibronectins and collagens has been extensively documented (1, 13, 18, 29). The repression of tropomyosin variants (15, 16, 22) and of the acidic isoform of actin (34) has also been described elsewhere. For this reason we did not characterize these polypeptides further. On the other hand, the repression of the 20-kDa quiescence-enriched polypetides (Fig. 1A) appears unique. In the following sections we describe the expression of the 20-kDa polypeptides in normal and transformed cells.

Expression of the quiescence-specific 20-kDa polypeptides. Normal quiescent CHM cells actively synthesized a pair of 20-kDa polypeptides (p20) which were missing in the autoradiographic pattern of transformed CHM cells (Fig. 1). This difference in the synthesis of p20 was reflected in the abundance of the polypeptides in the cell. Indeed, the p20 polypeptides were among the most prevalent proteins of quiescent CHM cells, whereas they were undetectable in the stained pattern of transformed cells even when a sensitive silver staining protocol was used (data not shown; see Fig. 3B). Two separable p20 polypeptides were found in quiescent cells. The most acidic form (pI of 6.3) was always more actively synthesized and was predominant by mass. Partial digestion of the minor and major isoforms with the protease V8 indicated that they are structurally related (data not shown). We prepared a polyclonal antiserum specific for the major 20-kDa polypeptide (serum 601; see Materials and Methods) and investigated the expression of this isoform in CHM cells. The results of these analyses apply to the minor p20 polypeptide as well since the expression of both minor and major forms appears to be coordinate (data not shown).

The autoradiographic patterns of metabolically labeled

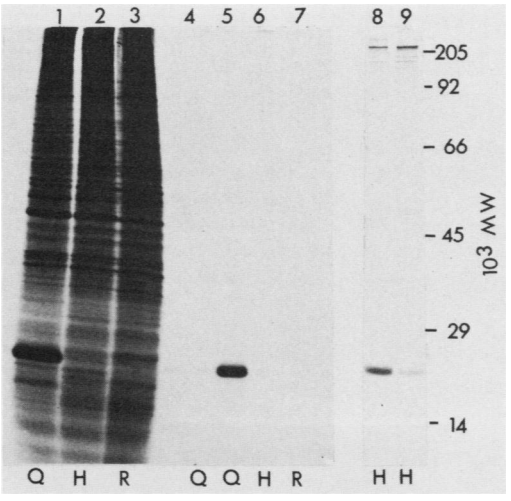

FIG. 2. Analysis of the quiescence-specific p20 protein. The p20 quiescence-specific polypeptide was immunoprecipitated from samples of radiolabeled proteins synthesized by normal quiescent (Q), normal hormone-stimulated (H), and RSV-transformed (R) CHM cells (lanes 5, 6, and 7, respectively). The total, nonprecipitated samples are shown in lanes 1, 2, and 3. Lane 4, Quiescent cell sample analyzed with the preimmune serum; lanes 8 and 9, a 10-fold excess of radiolabeled proteins synthesized by hormone-stimulated cells was used for immunoprecipitation by the p20 antiserum. The quiescent cells were labeled at a density of 9×10^5 cells per 35-mm dish (lanes 1, 4, and 5). The normal hormone-stimulated cells were labeled at 3×10^6 (lanes 2, 6, and 8) or 2×10^6 (lane 9) cells per 35-mm dish. The RSV-transformed cells were labeled at a density of 6.5×10^6 cells per 35-mm dish (lanes 3 and 7).

proteins synthesized by normal quiescent, normal hormone-stimulated, and RSV-transformed CHM cells are shown in Fig. 2 (lanes 1, 2, and 3, respectively). The synthesis of p20 in the three samples was analyzed by immunoprecipitation with the 601 serum (lanes 5, 6, and 7, respectively). The quiescent cell sample was also analyzed with the corresponding preimmune serum (Fig. 2, lane 4). The results of the immunoprecipitation analysis confirmed that p20 was predominantly synthesized by quiescent CHM cells. Longer exposure of the autoradiograph shown in Fig. 2 revealed a weak signal in lane 6 (hormone-stimulated cells) and no detectable signal in lane 7 (transformed cells). The presence of p20 synthesis in hormone-stimulated CHM cells (confirmed by two-dimensional gel analysis) was puzzling and first appeared to be variable in intensity from sample to sample. Subsequent analyses indicated that dense confluent CHM cells, even when stimulated by EGF and insulin, synthesized a low but detectable level of p20. p20 was immunoprecipitated from EGF-insulin-treated CHM cells seeded at 3×10^6 and 2×10^6 cells per 35-mm dish (Fig. 2, lanes 8 and 9, respectively). In both samples, p20 was detectably synthesized at levels determined by cell density. Subconfluent EGF-insulin-treated cells did not synthesize any detectable level of p20 (data not shown), whereas dense confluent cells synthesized low but increasing levels of the protein. In the samples analyzed in lanes 8 and 9, a 10-fold excess of radioactively labeled proteins was used for the immunoprecipitation, indicating that the levels of p20 synthesis by confluent hormone-stimulated CHM cells was low when compared with that by quiescent cells. Transformed CHM cells grown to an even higher cell density (lane 7) did not synthesize a detectable level of p20.

p20 accumulated in the culture medium. We found that p20 could be recovered from the culture medium. We examined the radiolabeled proteins collected from the culture medium of quiescent cells (Fig. 3A, lanes 1, 4, and 5), EGF-insulin-treated cells (lanes 2 and 6), and RSV-transformed cells (lanes 3 and 7). An equal number of counts was loaded in each lane (complete samples, lanes 1, 2, and 3) or analyzed by immunoprecipitation with the 601 serum (lanes 5, 6, and 7). The quiescent cell sample was also analyzed with the preimmune serum (lane 4). As in cell lysates, p20 represented a major synthesis product of quiescent CHM cells and was almost undetectable in EGF-insulin-treated or RSV-infected cells. A weak signal could be detected in lane 6 (EGF-insulin-treated cells) after longer exposure of the autoradiograph.

The analysis of complete samples in lanes 1, 2, and 3 revealed significant differences in the patterns of proteins shed in the medium. For instance, a 66-kDa polypeptide was prominently synthesized by transformed cells and, to a lesser extent, by EGF-insulin-treated cells. Senger et al. (30) have also reported the unique synthesis of secretory proteins of about 60 kDa by transformed cells. Differences among quiescent, hormone-stimulated, and RSV-transformed CHM cells were more striking among the proteins recovered from the culture medium than those observed in total cell lysates.

The accumulation of p20 in the medium was also analyzed by Western blotting (Fig. 3B). Equal fractions of the medium were loaded onto the gel, transferred to a nitrocellulose sheet, and examined with the 601 antiserum. A strong signal was detected only in the quiescent cell sample (lane 1). Our assay indicated that, by mass, the amount of p20 in the

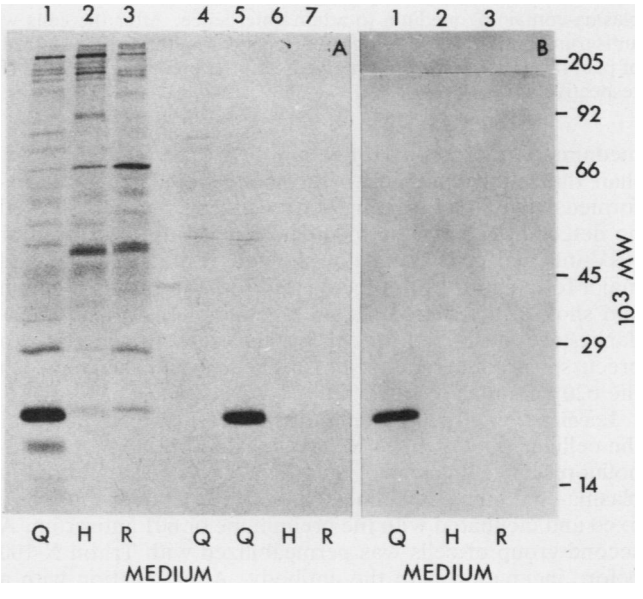

FIG. 3. Accumulation of p20 in the culture medium. (A) The pattern of radioactively labeled proteins shed into the culture medium of quiescent (Q), hormone-stimulated (H), and RSV-transformed (R) CHM cells is shown in lanes 1, 2, and 3, respectively. The immunoprecipitation of p20 from the three samples is shown in lanes 5, 6, and 7. Lane 4, The quiescence medium sample analyzed with the preimmune serum. (B) Abundance of p20 recovered from the culture medium of quiescent, hormone-stimulated, and RSV-infected cells (lanes 1, 2, and 3, respectively) was examined by the Western immunoblot techniques. An equal fraction of the proteins recovered from the culture medium was analyzed.

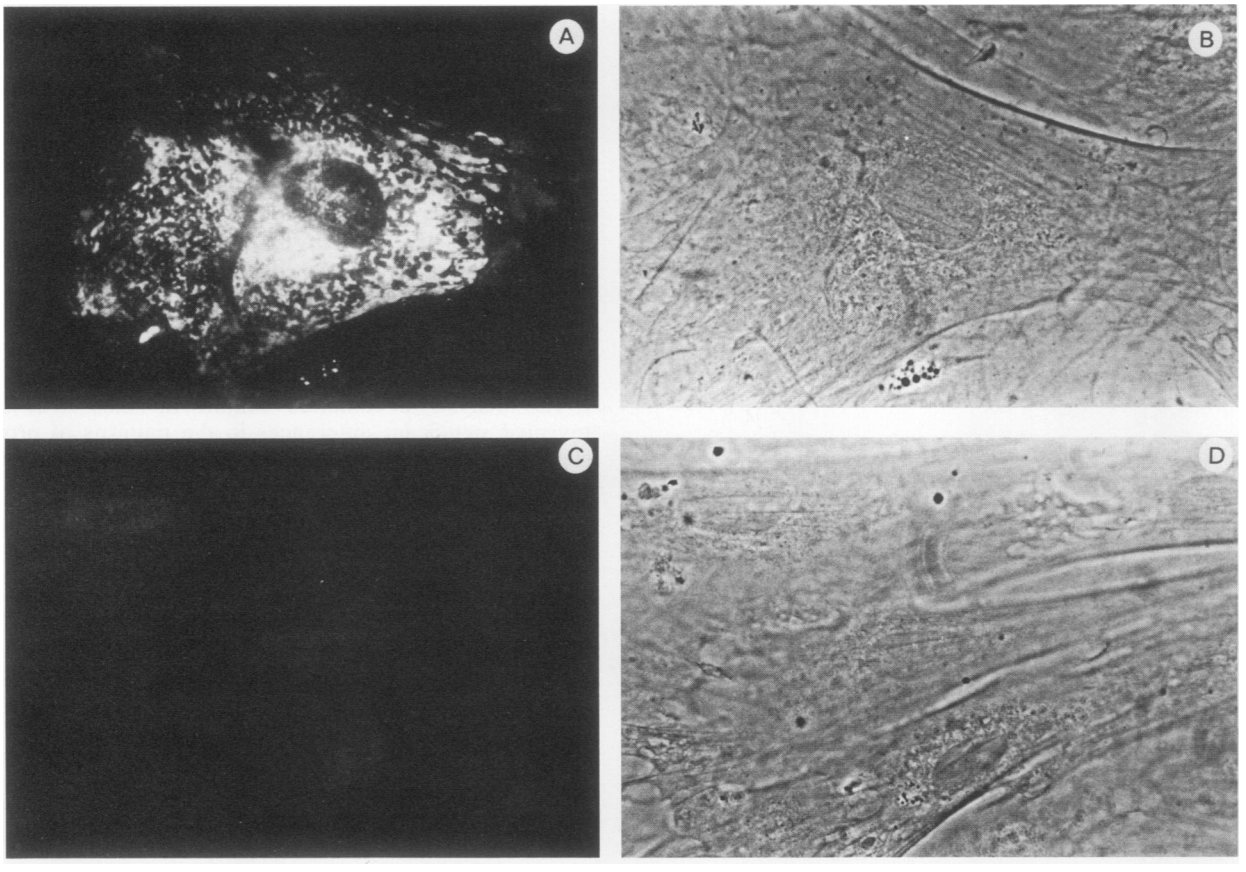

FIG. 4. Localization of p20 by immunofluorescence in quiescent CHM cells. CHM cells were seeded on cover slips and cultured in plasma-containing medium to attain quiescence. After the cells were fixed, permeabilized with Triton X-100, and incubated with the p20 antiserum, a fluorescein-conjugated secondary antibody was added to detect the 20-kDa immune complex. (A) Immunofluorescence pattern of p20; (C) staining pattern obtained with the preimmune serum; (B) and (D) phase-contrast photographs of cells shown in panels A and C, respectively.

medium of quiescent CHM cells was at least 10-fold greater than that in the medium of hormone-stimulated or transformed cells. Silver staining of two-dimensional gels showed no detectable p20 in the medium of EGF-insulin-treated or RSV-infected cells (data not shown). Both the minor and major forms of p20 were recovered from the culture medium and showed the same unequal labeling intensity and abundance level described for cell lysates (data not shown). No precursor-product relationship has been established between the p20 variants.

Localization of p20 by immunofluorescence. The nature of the cellular pool of p20 was investigated by indirect immunofluorescence analysis of quiescent CHM cells cultured in plasma-containing medium. Cells seeded on cover slips were fixed and incubated with the preimmune or 601 antiserum. A second group of cells was permeabilized with Triton X-100 before incubation with the antibody. After reaction with a fluorescein-conjugated secondary antibody, the localization of p20 was observed by fluorescence microscopy. No staining was detected in cells not treated with Triton X-100, indicating that the p20 pool was not associated with the external cell surface. The staining pattern observed in permeabilized cells is shown in Fig. 4A. The protein was distributed throughout a large portion of the cytoplasm, perhaps in a lamellar compartment, as suggested by the discrete "patches" of staining (Fig. 4A). We do not know if the staining pattern corresponded to the endoplasmic reticulum. Staining with the preimmune serum resulted in no significant signal (Fig. 4C). We conclude from this analysis that the cellular pool of p20 is internal.

Expression of p20 is density dependent. High cell density (confluence) markedly determined the expression of p20 in EGF-insulin-treated cells (Fig. 1). Cell density also influenced p20 synthesis in quiescent cells (Fig. 5). CHM cells were seeded at different densities in plasma-containing medium and metabolically labeled with L-[^{35}S]methionine, as described in Materials and Methods. The culture medium was collected, and a cell lysate was prepared for protein analysis by SDS-polyacrylamide gel electrophoresis and for immunoprecipitation with the 601 antiserum. Sparse CHM cells synthesized significant but reduced levels of p20 compared with levels synthesized by dense confluent cells (Fig. 5). This was true for p20 associated with the cell (Fig. 5A) or recovered from the medium (Fig. 5B). On the basis of the number of counts immunoprecipitated, we determined that as much as 5% of total protein synthesis was devoted to p20 in dense quiescent CHM cells. This level was roughly 20-fold greater than that of p20 synthesis in sparse cells (data not shown).

Other polypeptides were also influenced by cell density. The putative acidic actin isoform and tropomyosin variants missing in transformed cells were synthesized at higher levels in sparse cells and only marginally in dense cells (Fig. 5A; data not shown). Changes in the expression of other

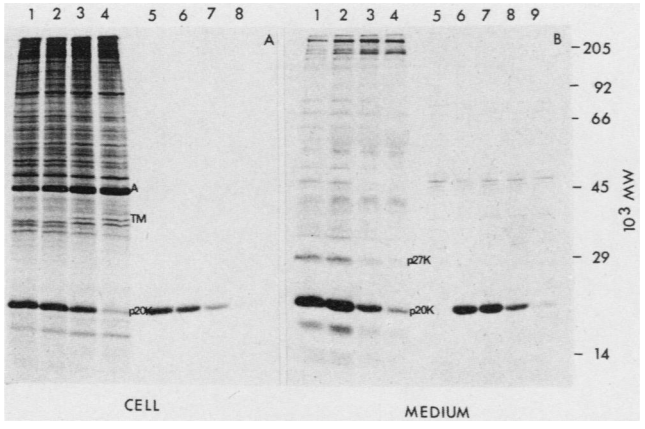

FIG. 5. Influence of cell density of p20 expression. (A) CHM cells seeded at different cell densities were metabolically labeled with L-[^{35}S]methionine in plasma-containing medium, and the proteins were analyzed by polyacrylamide gel electrophoresis. The patterns for cells seeded at 9, 6, 4, and 2×10^5 cells per 35-mm dish are shown in lanes 1 through 4, respectively. Under these conditions, cells at a density of 6×10^5 cells per 35-mm dish were confluent and cells at a density of 2×10^5 cells per 35-mm dish were sparse. Lanes 5 through 8, p20 was immunoprecipitated from the four samples and analyzed in parallel. (B) The radiolabeled proteins recovered from the medium of cells described above were examined. The total proteins shed by cells seeded at 9, 6, 4, and 2×10^5 cells per 35-mm dish are shown in lanes 1 through 4, respectively. p20 is shown after immunoprecipitation in lanes 6 through 9. Lane 5, The medium of dense cells was examined with the preimmune serum. Proteins indicated by symbols are described in the text.

secretory proteins were also observed. A 27-kDa polypeptide (Fig. 5B) showed a pattern of expression comparable to that of p20. It was more actively synthesized at high cell density (Fig. 5B) and was also repressed in transformed cells (data not shown). As evidenced by data shown above, this 27-kDa polypeptide was not recognized by the 601 antiserum.

Kinetics of repression of p20 synthesis. We stimulated quiescent CHM cells with EGF (100 ng/ml) and insulin (10,000 ng/ml) and examined the expression of p20 at different times thereafter. In this analysis subconfluent cells were metabolically labeled for 1 h after 5, 15, or 25 h of EGF-insulin addition to the plasma-containing medium. The results of immunoprecipitations performed on cell lysates are shown in Fig. 6A. The synthesis of p20 was maintained for the first 5 h of stimulation but decreased considerably between 5 and 15 h. The same was true for newly synthesized p20 recovered from the culture medium.

We addressed the same question with cells infected with a temperature-sensitive RSV mutant, NY 72-4 (24). CHM cells infected with the temperature-sensitive mutant were cultured at the nonpermissive temperature of 41°C in plasma-containing medium. Under these conditions, the NY 72-4-infected CHM cells do not divide and appear morphologically normal (4). We monitored the synthesis of p20 in cells maintained at 41°C or transferred to the permissive temperature of 35°C for various periods of time. The results of the immunoprecipitation analysis are shown in Fig. 6B. Under these conditions, p20 synthesis did not change for several hours and decreased significantly only after 24 h at the permissive temperature. In this series of experiments the initiation of cell division and morphological changes also occurred slowly and may account for the kinetics of p20 repression (data not shown). Translation of polyadenylated RNA purified from quiescent and transformed CHM cells indicated that the level of p20 synthesis reflected the abundance of translatable RNA present in the cell (data not shown); the slow kinetics of p20 repression may have been determined by the decay of the p20 mRNA. On the basis of the analyses shown in Fig. 6A and B, we conclude that the repression of p20 synthesis is a slow process.

Expression of p20 in CEF. To determine whether p20 synthesis is restricted to CHM cells, we labeled normal CEF in medium containing serum or plasma. We found that, more or less independently of the medium (serum or plasma; in SC-12 or DMEM), CEF expressed p20 principally at confluence. For the analysis shown in Fig. 7, normal CEF were grown to various densities in DMEM containing serum and were metabolically labeled with L-[^{35}S]methionine for 90 min. Protein samples were prepared as described above and analyzed with the 601 antiserum. The results of the immunoprecipitation of cell lysates and culture media are shown in Fig. 7A and B, respectively. In CEF the synthesis of p20 was also density dependent but was negligible in subconfluent cells. The same was true for cells cultured in plasma and for primary or late-passage cultures. Under the conditions of labeling, about 25% of the newly synthesized p20 was associated with the cell fraction of CEF, whereas as much as

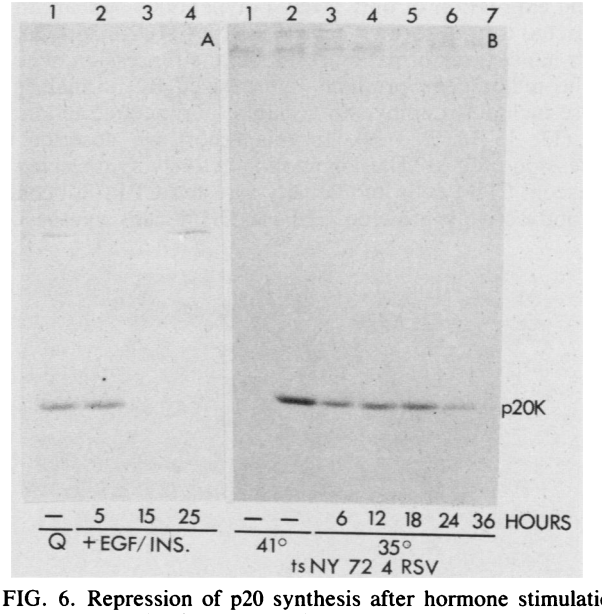

FIG. 6. Repression of p20 synthesis after hormone stimulation and pp60$^{v\text{-}src}$ activation. (A) Quiescent CHM cells seeded at a density of 3×10^5 cells per 35-mm dish (subconfluence) were stimulated with 100 ng of EGF per ml and 10,000 ng of insulin (INS) per ml. The cells were labeled for 1 h with L-[^{35}S]methionine at different times after stimulation. The synthesis of p20 was analyzed after immunoprecipitation of cell lysates prepared from quiescent cells (lane 1) and cells stimulated for 5, 15, and 25 h (lanes 2, 3, and 4, respectively). (B) CHM cells infected with the temperature-sensitive (ts) mutant RSV NY 72-4 were cultured in plasma-containing medium at the nonpermissive temperature of 41°C. To induce transformation, the cells were transferred to the permissive temperature of 35°C for increasing periods of time. After transfer, the cells were metabolically labeled with L-[^{35}S]methionine and analyzed for p20 synthesis by immunoprecipitation with the 601 p20 antiserum. The result of this immunoprecipitation performed on cell lysates is shown for cells at 41°C (lane 2) and cells transferred to 35°C for 5, 12, 18, 24, and 36 h (lanes 3 to 7, respectively). Lane 1, The 41°C sample was analyzed with the preimmune serum.

50% of the labeled p20 was recovered from CHM cells. Even in dense confluent CEF (cell fraction), radiolabeled p20 accounted for about 0.2 to 0.5% of the counts incorporated into trichloroacetic acid-precipitable proteins (compared with 5% in CHM cells). Western blotting indicated that p20 accumulated substantially in the medium of dense CEF (data not shown).

The synthesis of p20 by CEF infected with the temperature-sensitive mutant RSV NY 72-4 was also examined. Infected CEF were grown to confluence in serum-containing DMEM at the nonpermissive temperature of 41°C and then transferred to 35°C for increasing periods of time. At the end of the incubation period, they were metabolically labeled with L-[^{35}S]methionine and processed for protein analysis, as described above. The immunoprecipitation of p20 from the culture medium (Fig. 8) demonstrated that p20 synthesis was considerably repressed at the permissive temperature of 35°C. The same was true for the cell-associated p20 (data not shown). The decrease became apparent between 4 and 12 h after the temperature shift. Densitometric scanning of the autoradiograph shown in Fig. 8 indicated that after 24 h at 35°C, the NY 72-4-infected CEF synthesized p20 at only 20% of the level observed at the nonpermissive temperature (data not shown).

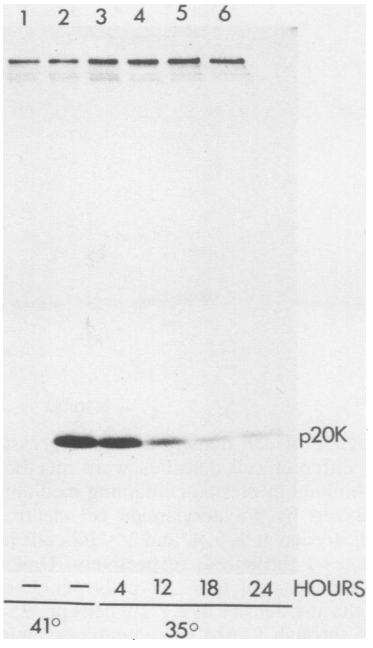

FIG. 8. Repression of p20 synthesis in CEF infected with temperature-sensitive RSV NY 72-4. CEF infected with the temperature-sensitive mutant RSV NY 72-4 were grown to confluence in serum-containing medium at the nonpermissive temperature of 41°C. After radiolabeling of the CEF, p20 recovered from the medium was analyzed by immunoprecipitation. Lanes 3 to 6, p20 synthesis analyzed in RSV NY 72-4-infected CEF transferred to the permissive temperature for 4, 12, 18, and 24 h, respectively; lanes 1 and 2, the preimmune and 601 p20 antisera, respectively, were used to immunoprecipitate p20 from the medium of nontransformed RSV NY 72-4-infected CEF maintained at the nonpermissive temperature.

DISCUSSION

The expression of only a few polypeptides appears to be restricted to transformed cells (Fig. 1; 9, 11, 20, 26). On the other hand, transformation results in the repression of some of the major gene products synthesized by normal cells. These include tropomyosin variants, fibronectin, and collagen (13, 15–18, 22, 29). In this report we describe the repression of a 20-kDa polypeptide actively synthesized by quiescent CHM cells and density-arrested CEF. Subconfluent and actively growing CEF or CHM cells synthesized little p20. Increasing amounts of the polypeptide were synthesized when growing cells reached confluence (high density). Thus, quiescence per se was apparently the primary factor determining the expression of p20. Transformed cells, which were not density arrested and were actively growing even in low concentrations of serum or plasma, did not synthesize a detectable level of the protein (Fig. 2). Finally, quiescent CHM cells entering the cell cycle after hormone stimulation or after pp60$^{v\text{-}src}$ activation slowly but considerably repressed the synthesis of p20 (Fig. 6).

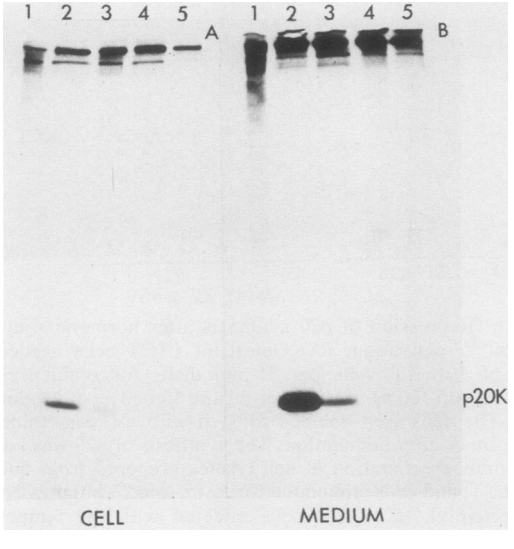

FIG. 7. Expression of p20 in normal CEF. CEF were grown to different cell densities in serum-containing medium and labeled with L-[^{35}S]methionine for 90 min. The synthesis of p20 was examined by immunoprecipitation of cell lysates (A) and culture media (B). Immunoprecipitated p20 is shown for cells at a density of 3.5×10^6 (dense confluent), 1.5×10^6 (confluent), 6×10^5 (subconfluent), and 3×10^5 (sparse) cells per 35-mm dish in lanes 2 to 5, respectively. Lane 1, Samples from dense CEF were analyzed with the preimmune serum.

We chose to investigate gene expression in CHM cells because of their growth characteristics. The cells are proliferatively quiescent in a medium of physiological composition supplemented with plasma (3, 6). The CHM cells proliferate upon stimulation by a wide variety of growth factors or hormones but apparently do not respond to the low levels of endogenous EGF and insulinlike growth factors (IGF I and II) present in plasma (5). These characteristics are favorable for the investigation of cell replication and cell quiescence under physiological conditions. The synthesis of p20 is not restricted, however, to CHM cells. CEF, which are not proliferatively quiescent in a plasma-containing medium (3), expressed p20 at confluence (Fig. 7). Serum or plasma depletion (starvation) does not induce p20 synthesis in CHM cells or CEF (unpublished results). Hence, not all conditions of growth arrest lead to the expression of p20. Recently, Zullo et al. (36) reported the delayed induction of the β-interferon and (2′,5′)-oligoadenylate synthetase genes

in BALB/c cells stimulated by platelet-derived growth factor. This late induction, which is now hypothesized to be part of a feedback mechanism controlling cell growth, was not observed in subconfluent cells stimulated by platelet-derived growth factor (36). Hence, the molecular biology of growth control may differ in cells arrested at confluence or subconfluence, with or without serum depletion. It is interesting to speculate that in confluent CHM cells quiescence is the result of two factors, i.e., the lack of response to the low levels of endogenous growth factors present in plasma and the inhibition provided by cell confluence. Thus, in this cell system, the expression of genes induced at quiescence, such as the p20 gene, may well be amplified. With this hypothesis in mind, it would also be interesting to examine interferon expression in quiescent CHM cells.

Several investigators have hypothesized that the repression of gene products synthesized by normal cells is necessary for the genesis of the transformed phenotype. Hendricks and Weintraub (15) proposed that the inactivation of synthesis of tropomyosins may hinder the stable assembly of actin filaments and result in the disruption of the actin cables observed in transformed cells. Yamada et al. (35) and Ali et al. (2) reported that the addition of exogenous fibronectin partly restores the normal morphology and adhesiveness of transformed cells. The significance of p20 repression remains to be determined. Because p20 accumulates in the medium, its function is amenable to experimental analysis. We are in the process of examining the effects of exogenously added p20 on the morphology and growth rates of dividing and transformed cells.

Many aspects of p20 expression remain uncharacterized. There was a significant fraction of p20 that was located intracellularly (Fig. 3). The electrophoretic characteristics of the protein(s) collected from the culture medium and associated with the cell were indistinguishable on two-dimensional gels. In CHM cells much of the intracellular pool of p20 can be chased into the culture medium, but radiolabeled p20 can still be detected in the cell fraction after chase (unpublished results). Under the same conditions of labeling, newly synthesized p20 partitioned differently between the cellular and extracellular fractions in CHM cells and CEF. Clearly, much remains to be investigated in the processes leading to the accumulation of p20 in the culture medium. By translating polyadenylated RNA in a reticulocyte cell-free translation system, we found that a 22-kDa protein is immunoprecipitated by the 601 antiserum. This observation suggests that the primary p20 translation product includes a signal peptide. Our goal is to understand the expression of p20 in quiescent cells and to investigate the molecular events leading to its repression in their transformed counterparts. By doing so we hope to identify some of the molecular pathways affected by $pp60^{v\text{-}src}$ in the control of cell proliferation.

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