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Levels of translatable mRNAs for cell surface protein, collagen precursors, and two membrane proteins are altered in Rous sarcoma virus-transformed chick embryo fibroblasts

(transformation/LETS protein/cell-free translation/glucose regulated proteins)

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ABSTRACT **Transformation of chick embryo fibroblasts by Rous sarcoma virus results in decreased amounts of a major cell surface protein and of collagen. To determine the mechanism accounting for the decreased production of these proteins, we have measured the relative amounts of functional mRNAs for these and other transformation-sensitive proteins. Total cellular RNAs extracted from normal cells and from cells transformed by the Schmidt-Ruppin strain of Rous sarcoma virus were translated in a cell-free system derived from wheat germ. Analysis of the *in vitro* translation products of RNAs from normal and transformed chick embryo fibroblasts shows a 5-fold reduction in the translatable mRNA for cell surface protein and a 10-fold reduction in translatable mRNA for two collagen precursors. In addition, increases in functional mRNA are observed for myosin and for two membrane polypeptides with molecular weights of 95,000 and 78,000; the latter two proteins increase on transformation, but the increases are in large part secondary to the depletion of glucose from the medium of transformed cells. Our data suggest that some of the major cellular changes induced by oncogenic viruses are due to changes in the activity of specific cellular genes.**

Neoplastic transformation of fibroblastic cells results in decreased amounts of a major cell surface protein (CSP), also called large external transformation-sensitive protein (LETS protein) (1–8), and in decreased amounts of a major extracellular protein, collagen (9–11). The altered shape and adhesive properties of transformed chick embryo fibroblasts (CEF) and some other cells have been attributed to their diminished CSP content (7, 12, 13). Furthermore, loss of CSP has been correlated with the ability of many transformed cell lines to form tumors (14). Several mechanisms have been proposed to explain why transformed cells have less CSP. These include decreased biosynthesis (4), increased degradation due to the increased proteolytic activity of transformed cells (3, 15), and increased release from the cell surface into the medium (16). Recent experiments by Olden and Yamada (17) using metabolic labeling techniques indicate that a decrease in the synthetic rate of CSP is the major mechanism for the reduction of CSP in transformed CEF. Peterkofsky (10) and Levinson *et al.* (11) have shown that the decreased collagen content of transformed cells may also be attributed to decreased synthesis. Other proteins have been found to increase after transformation of CEF; among these are two glucose-regulated membrane proteins with molecular weights of 95,000 (GRP-95) and 78,000 (GRP-78) (4, 18–20).

In this study, we have measured the relative amounts of translatable mRNA coding for these transformation-sensitive proteins by extracting RNA from normal and transformed CEF and translating the mRNA in a cell-free system prepared from wheat germ. The translation products were analyzed by immunoprecipitation, collagenase digestion, and sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis. We present evidence that Rous sarcoma virus-transformed CEF contain less translatable mRNA for CSP and for two collagen precursors than do normal cells. Our experiments also show that transformation results in increased functional mRNA for GRP-95 and GRP-78.

MATERIALS AND METHODS

Cells. CEF were prepared and propagated in GM medium at 39° as described by Vogt (21). Second or third passage cells (1×10^7) were planted in 150-mm dishes (Falcon) and grown for 2 days before harvesting. Cells were transformed with either the Schmidt-Ruppin (SR) strain of Rous sarcoma virus or with a temperature-sensitive mutant of that strain (SR-T5) that was isolated and characterized by Martin (22).

Extraction of RNA. Total cellular RNA was isolated from normal and transformed CEF by a modification (23) of the guanidine extraction method (24). This procedure was developed in the laboratory of W. Rutter (University of California, San Francisco) and was communicated to us by A. Burns, R. Deeley, and B. Paterson (National Institutes of Health), with several modifications. Cells grown in 10 150-mm dishes were solubilized in 40 ml of a solution containing 8 M guanidine hydrochloride/10 mM sodium acetate, pH 5.2/1 mM dithiothreitol and homogenized in a Dounce homogenizer with 10 strokes each of the loose and tight pestles. RNA was precipitated from the homogenate by incubation with a one-half volume of ethanol at −20° for 30 min. The precipitate was dissolved in 10 ml of a solution containing 8 M guanidine hydrochloride/10 mM sodium acetate, pH 5.2/1 mM dithiothreitol/20 mM EDTA, and the RNA was precipitated three more times with ethanol as above. The RNA was then dissolved in 10 ml of 20 mM EDTA, pH 7.0, and extracted three times against an equal volume of a chloroform/isobutanol mixture of 4:1 (vol/vol). Finally, the RNA was precipitated overnight at −16° in 3 M sodium acetate, pH 5.2; washed in 3 M sodium acetate and in 70% ethanol; reprecipitated in 0.1 M sodium acetate, pH 5.2, with 2.5 volumes of ethanol at −50° for 1 hr; and washed several more times in 70% ethanol. The average yield of extracted RNA was 0.35 mg per dish, as mea-

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Abbreviations: CSP, cell surface protein; CEF, chick embryo fibroblasts; GRP, glucose regulated protein; LETS protein, large external transformation-sensitive protein; SR, Schmidt–Ruppin strain of Rous sarcoma virus; SR-T5, temperature-sensitive mutant of SR; $NaDodSO_4$, sodium dodecyl sulfate.

sured by absorption at 260 nm. This was approximately 65% of the yield of RNA extracted by the phenol/$NaDodSO_4$ procedure (25); however, the yield of mRNAs coding for proteins with molecular weight greater than 100,000 (as assayed by translation *in vitro* as described below) was greatly increased by the guanidine extraction procedure.

Translation of CEF RNA in a Wheat Germ S30. The preparation of the wheat germ S30 fraction and conditions of the translation assay have been described by Roberts and Paterson (26), with the following modifications, suggested to us by J. Gordon and B. Paterson (National Institutes of Health): each 50-μl reaction contained 500 μg of S30 protein/4 μM L-[^{35}S]methionine (specific activity, 400 Ci/mmol; Amersham)/95 mM KCl/1 mM Mg acetate/0.6 mM spermidine/12–15 μg of total cellular RNA. The incubation was for 3 hr at 21°. Total protein synthesis was determined by precipitation of 5-μl aliquots of the reaction mixtures with 1 ml of 10% trichloroacetic acid for 15 min at 100° and then for 10 min on ice, followed by collection of the precipitates on nitrocellulose filters.

Preparation of Antibodies. Goat antisera were raised against CSP from CEF and against cyclic AMP receptor protein from *Escherichia coli;* IgG fractions were prepared and affinity-purified as described (27–29).

Immunoprecipitation of Cell-Free Translation Products. Immunoprecipitation of translation products was performed as described, with minor modifications (17). At the end of the 3-hr protein synthesis incubations, 5 μg of unlabeled CSP was added to each reaction mixture as carrier, samples were diluted by addition of 1 ml of a solution containing 50 mM sodium phosphate, pH 11/1% Triton X-100/2 mM phenylmethylsulfonyl fluoride to solubilize newly synthesized CSP (17), and then the samples were neutralized by the addition of 1 ml of 100 mM sodium phosphate, pH 7.0/100 mM NaCl/1% Triton X-100/2 mM phenylmethylsulfonyl fluoride. Affinity-purified antibodies against CSP or cyclic AMP receptor protein (30–40 μg) were added and samples were incubated for 1 hr at 20°; 0.5–0.7 ml of rabbit antiserum prepared against goat IgG (1.5–2.1 mg of specific antibody, Miles Laboratories) was added and the incubation was continued for 30 min at 20°. Precipitates were collected by centrifugation for 10 min at 12,000 × *g* and washed twice with Dulbecco's phosphate-buffered saline with 1% Triton X-100 and then once with Dulbecco's calcium and magnesium free phosphate-buffered saline.

Collagenase Digestion of Cell-Free Translation Products. At the end of the translation incubations, the reaction mixtures were made 1 mM in $CaCl_2$ and purified collagenase was added to a concentration of 13 μg/ml. Samples were incubated for 10 or 30 min at 37°.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Products from the cell-free protein synthesis reactions, immunoprecipitates, and collagenase digestion reactions were identified by $NaDodSO_4$/polyacrylamide gel electrophoresis according to the procedure of Laemmli (30). Samples were dissolved in 100 mM Tris, pH 6.8/2% $NaDodSO_4$/4 M urea and placed in a boiling-water bath for 3 min; dithiothreitol was added to a concentration of 100 mM and samples were incubated for 1.5 min at 100° and then for 30 min at 37°. Samples were applied to 8% gels with 4% stacking gels and electrophoresed for 6 hr at 25 mA.

Autoradiography. Gels were treated with a 20% solution of 2,5-diphenyloxazole in dimethylsulfoxide according to the procedure of Laskey and Mills (31) and dried in a Bio-Rad slab-gel drier. Kodak RP/2 Royal X-Omat film was exposed for varying periods of time at −70°. Autoradiographs were scanned with a Joyce-Loebl microdensitometer and areas under the peaks were determined with an electronic planimeter (Numonics Corp.).

RESULTS

CEF RNA Directs the Synthesis of CSP, Collagen Precursors, and Myosin in the Wheat Germ S30. Initial experiments indicated that total cellular RNA isolated by guanidine extraction stimulates incorporation of [^{35}S]methionine into acid-insoluble material 2- to 5-fold over the endogenous activity of the wheat germ S30. All preparations of RNA, from both normal and transformed cells, showed comparable activity in terms of total protein synthesis. The incorporation was directly proportional to the RNA concentration from 2 to 10 μg of added RNA; above 10 μg of RNA, no further increase was observed (data not shown). Saturating levels of 12–15 μg of RNA were used in all subsequent experiments. The autoradiograph in Fig. 1 (lane 2) shows the *in vitro* translation products from syntheses directed by RNA isolated from normal CEF; this pattern was identical at all RNA concentrations examined. The products of translation *in vitro* are similar to total proteins extracted from [^{35}S]methionine-labeled cells (data not shown). Even high-molecular-weight proteins, including CSP and myosin, were synthesized *in vitro*, as indicated by coelectrophoresis of these polypeptide chains with the authentic proteins. It should be noted, however, that the high-molecular-weight polypeptides were synthesized in reduced amounts in the wheat germ S30 compared to lower-molecular-weight proteins. Furthermore, direct gel analysis of the translation products sometimes failed to show CSP in the expected quantities, perhaps because of problems of CSP solubility or electrophoresis in the presence of wheat germ S30.

To identify CSP and quantitate the amount of CSP synthesized *in vitro*, the reaction mixtures were immunoprecipitated with affinity-purified antibody against CSP. Fig. 1 (lane 7) shows that this treatment greatly enriched a protein band with the electrophoretic mobility of CSP. A control experiment was done in which affinity-purified antibodies prepared against an *E. coli* protein (cyclic AMP receptor protein) were used (Fig. 1, lane 8). There was no significant precipitation of CSP or other high-molecular-weight proteins. Some low-molecular-weight proteins, including one with the electrophoretic mobility of actin, appeared to precipitate nonspecifically, since they were observed as products of immunoprecipitation with antibodies against CSP (Fig. 1, lane 7), cyclic AMP receptor protein (Fig. 1, lane 8), and myosin (data not shown).

In addition to CSP, two other proteins with high molecular weights (180,000–190,000 and 150,000) were enriched in the immunoprecipitate produced by antibodies against CSP. Since the CSP used to prepare the antibodies was a homogeneous protein and the antibodies were purified by CSP affinity chromatography, it is very unlikely that these antibodies were contaminated with antibodies to other cellular proteins. It seems possible, therefore, that either the two additional proteins share antigenic sites with CSP or they readily associated with CSP. We favor the latter interpretation, because it has recently been observed that the LETS protein of BHK cells interacts with collagen (29). Our evidence suggests that these two proteins are collagen precursors.

To identify the presence of collagen precursors among the *in vitro* synthesized polypeptides, the CEF RNA-dependent translation products were treated with purified collagenase. It can be seen in Fig. 1 (lanes 3 and 4) that collagenase digested only two polypeptides, with apparent molecular weights of 180,000–190,000 and 150,000; the larger of these two bands

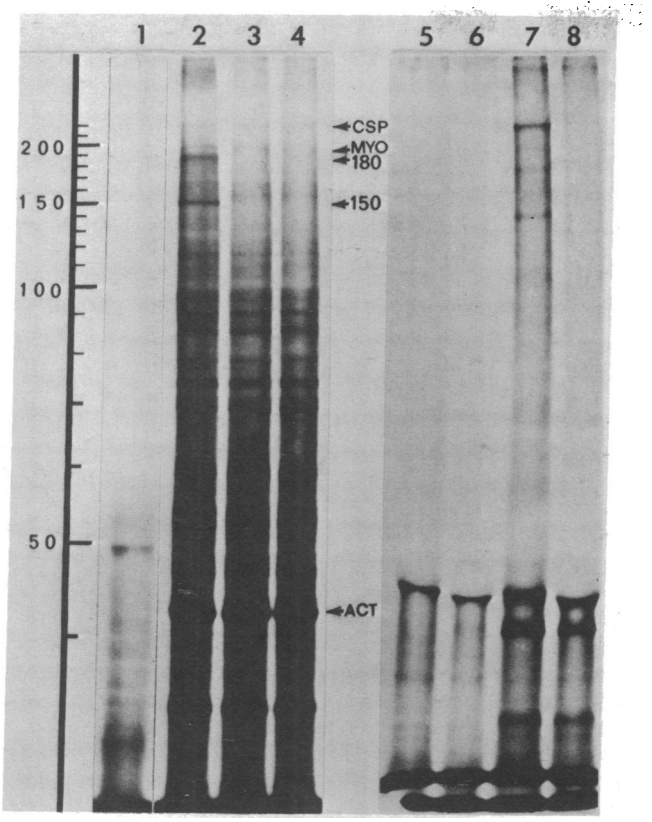

FIG. 1. Products of cell-free translation reactions directed by RNA isolated from normal chick embryo fibroblasts. Translation reactions were performed as described in *Materials and Methods*, with no exogenous RNA or with 12 μg of total RNA isolated from normal CEF. Products of *in vitro* translation reactions were either analyzed directly by discontinuous gel electrophoresis; digested with collagenase (13 μg/ml) for analysis of collagen precursors, followed by $NaDodSO_4$/gel electrophoresis; or immunoprecipitated with anti-CSP or antibodies against cyclic AMP receptor protein, followed by $NaDodSO_4$/gel electrophoresis. Eight percent of the total translation reactions and the collagenase digested translation reactions were applied to the gels; 25% of the immunoprecipitated translation reactions were applied to the gels. (Lane 1) Translation products of endogenous (wheat germ) RNA. (Lane 2) Translation products of normal CEF RNA. (Lane 3) Translation products of normal CEF RNA digested with collagenase (13 μg/ml) for 10 min. (Lane 4) Translation products of normal CEF RNA digested with collagenase (13 μg/ml) for 30 min. (Lane 5) Immunoprecipitation with anti-CSP of translation products of endogenous RNA. (Lane 6) Immunoprecipitation with antibodies against cyclic AMP receptor protein of translation products of endogenous RNA. (Lane 7) Immunoprecipitation with anti-CSP of translation products of normal CEF RNA. (Lane 8) Immunoprecipitation with anti-cyclic AMP receptor protein of translation products of normal CEF RNA. Molecular weights were determined by electrophoresis of the following standards: myosin, 200,000; β subunit of RNA polymerase, 160,000; β′ subunit of RNA polymerase, 150,000; phosphorylase *a*, 94,000; α subunit of RNA polymerase, 90,000; albumin, 68,000; and ovalbumin, 43,000. Abbreviations: CSP, cell surface protein; MYO, myosin, ACT, actin.

often appeared as a doublet. These two protein bands coelectrophoresed with the two polypeptides that were present in the anti-CSP immunoprecipitate (Fig. 1, lane 7). The molecular weights of these polypeptides are similar to those of pro-α_1 (I) and pro-α_2 (I) collagen prepared in this laboratory by pulse-labeling CEF with [^{14}C]proline (data not shown).

Myosin was identified by immunoprecipitation with affinity-purified antibodies against fibroblast myosin (33). The product coelectrophoresed with authentic CEF myosin (data not shown). Thus, four specific large polypeptides were identified among the *in vitro* translation products from CEF RNA-directed reactions: CSP, two collagen precursors, and myosin.

Table 1. Relative quantities of translatable mRNAs in normal and transformed cells

Protein	Normal CEF	SR-transformed CEF ("fed")	SR-transformed CEF ("starved")
CSP	1.0	0.2	N.D.
Myosin	1.0	2.7	3.0
180,000*	1.0	0.1	<0.1
150,000*	1.0	0.1	<0.1
GRP-95	1.0	1.2	2.5
GRP-78	1.0	1.4	2.4
Actin	1.0	1.0	1.0

Autoradiographs from several experiments were scanned with a Joyce-Loebl microdensitometer and areas under the peaks were determined with an electronic planimeter. Autoradiographs were exposed for varying lengths of time, to ensure that the exposure times used for these calculations were within the linear response range for all the proteins measured. A value of 1.0 was assumed for the amount of each protein synthesized by mRNA from normal CEF; the amount of each protein synthesized by RNAs from "starved" and "fed" SR-transformed CEF was calculated relative to the normal value. Each number represents an average of three experiments, in which at least two different preparations of each type of RNA were utilized. All data were derived from the application of total translation products to $NaDodSO_4$/polyacrylamide gels, except data for CSP, which were derived from immunoprecipitation of translation reaction products with anti-CSP. Equal aliquots of the translation reaction mixtures, containing similar amounts of acid-insoluble counts, were applied to the gels. N.D., not determined.
* Number refers to the molecular weight of the protein.

CSP Synthesis Directed by RNAs from Normal and Transformed CEF. Translation reactions directed by RNAs isolated from normal and SR-transformed CEF were treated with anti-CSP; a 5-fold decrease in the amount of CSP synthesized was evident when the RNA was derived from transformed cells (Table 1; Fig. 2, lanes 1 and 2). In the same reactions, the majority of the polypeptide products were synthesized in equal amounts whether translation was directed by RNAs derived from normal or transformed cells. To correlate the reduction in mRNA activity for CSP synthesis more directly with viral transformation, activities of RNA preparations isolated from SR-T5-infected cells were examined. Cells infected with this mutant virus manifest a transformed morphology at 36.5° and a normal morphology at 41°. These cells were grown at the temperature permissive for transformation (36.5°) and either maintained at that temperature or shifted to the restrictive temperature (41°) 24 hr before harvesting.

Analysis of the translation products by immunoprecipitation showed a 4-fold increase in CSP mRNA activity in SR-T5-infected cells that were shifted to the restrictive temperature (Fig. 2, lane 4), relative to the cells maintained at the permissive temperature (Fig. 2, lane 3). Furthermore, when SR-T5-infected cells were grown at the restrictive temperature and then shifted to the permissive temperature 24 hr before harvesting, there was a 4-fold decrease in mRNA activity for CSP synthesis (data not shown). RNAs derived from normal cells that had been subjected to the same temperature shifts as the SR-T5 infected cells showed no changes in amount of CSP synthesized *in vitro* (data not shown).

Synthesis of Collagen Precursors Directed by RNAs from Normal and Transformed CEF. The two collagen precursors that appear in substantial amounts when normal RNA is

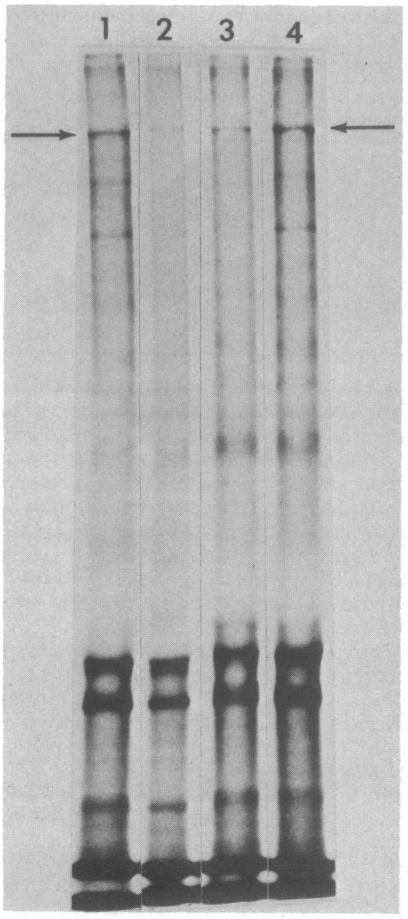

FIG. 2. Immunoprecipitation of CSP in translation reactions directed by RNAs isolated from normal and transformed CEF. Translation reactions were performed as described in *Materials and Methods*, with 12 μg of RNA from normal CEF (lane 1); SR-transformed CEF (lane 2); SR-T5 infected CEF maintained at 36.5°, the permissive temperature for transformation (lane 3); and SR-T5 infected CEF grown at 36.5° and shifted to the nonpermissive temperature (41°) 24 hr prior to harvesting (lane 4). Twenty-five percent of each immunoprecipitation reaction was applied to the gel. The arrows indicate the position of CSP.

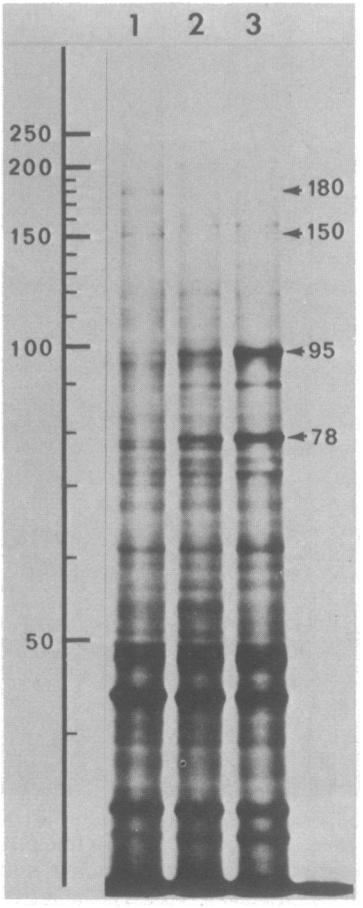

FIG. 3. Effect of glucose starvation on mRNA activity for GRP-95 and GRP-78. RNAs were isolated from normal CEF (lane 1) and from SR-CEF that had been grown in medium containing 24 mM glucose (lane 2) or medium containing 6 mM glucose (lane 3). Translation reactions were done as described in *Materials and Methods*, with 12 μg of added RNA. Eight percent of the total translation products was applied to the gel.

translated (Fig. 3, lane 1) are almost completely absent when the RNA is derived from SR-transformed cells (Fig. 3, lanes 2 and 3; Table 1). Further treatment of the latter translation reactions with collagenase leaves the protein pattern unchanged (data not shown).

Increase in Functional mRNAs Coding for Other Proteins on Transformation. The amount of several discrete translation products increased when the RNA was derived from transformed CEF; these products have apparent molecular weights of 200,000, 95,000, 87,000, 78,000, and 72,000 (Fig. 3, lanes 2 and 3). The 200,000-molecular-weight protein coelectrophoresed with myosin and could be immunoprecipitated with affinity-purified antibodies raised against fibroblast myosin (data not shown). The proteins with molecular weights of 95,000 and 78,000 had the same electrophoretic mobility as two major membrane proteins, GRP-95 and GRP-78, which have been observed to increase after transformation (4, 19, 20).

Glucose Regulation of Protein Synthesis. Other experiments from this laboratory have shown that the increase in transformed CEF of GRP-95 and GRP-78 can be almost completely prevented by maintaining glucose at normal levels with frequent additions of glucose to the medium (19, 20). A comparison was made of the translation products of RNAs derived from Rous sarcoma virus-transformed CEF that had been fed either high levels of glucose (24 mM) or normal levels of glucose (6 mM) in culture 18 hr before harvesting. Other studies in this laboratory have shown that SR-transformed CEF fed 6 mM glucose have almost completely exhausted the glucose in the medium by 18 hr (unpublished data). The data in Fig. 3 show that glucose starvation of SR-transformed CEF (lane 3) increased the amounts of translatable mRNA for both GRP-95 and GRP-78 2.5-fold above the levels found in normal cells and 2-fold above the levels found in glucose-fed SR-transformed CEF. Thus, the changes in GRP-95 and GRP-78 were largely secondary to the rapid depletion of glucose from the medium that occurs with transformed cells. Little change in the RNA activity for CSP and the collagen precursors was observed on glucose starvation or refeeding.

DISCUSSION

Transformation of cells by RNA tumor viruses alters the pattern of proteins associated with these cells, either by increasing or decreasing the amounts of cell-specific proteins, and promotes the synthesis of virus-specific polypeptides (1–11). To compare the levels of specific RNAs for these proteins in normal and transformed CEF, we have utilized a cell-free system from wheat germ. RNA has been extracted directly from cells by a method which protects mRNA against nuclease digestion and

thereby allows extraction of intact large mRNAs (23). We have examined seven different lots of wheat germ, and have found four that will translate these mRNAs into large polypeptides. Our results indicate that several of the changes in protein levels in transformed CEF are due to quantitative changes in functional messenger RNAs that code for those proteins.

We have examined the mRNA activities that direct the synthesis of CSP, a major cell surface glycoprotein, collagen precursors, and two membrane proteins. CSP has been identified as a product of translation *in vitro* of CEF RNA with specific immunoprecipitation and with electrophoresis in $NaDodSO_4$/polyacrylamide gels. Similarly, the collagen precursors have been shown to be products of translation *in vitro* by their specific sensitivity to digestion with purified collagenase. Our results clearly indicate that transformation of CEF with Rous sarcoma virus results in a considerable reduction of the functional mRNAs for CSP and the collagen precursors. Our data are in agreement with *in vivo* labeling studies showing that the synthesis of CSP (17) and of collagen (11) is decreased in CEF transformed by Rous sarcoma virus. Thus, the translatable mRNAs specifying two extracellular proteins that appear to have an important role in the interaction of normal fibroblasts with their neighboring cells and substratum are greatly reduced after transformation. The mechanism responsible for the decreased levels of translatable mRNAs needs further investigation; possible mechanisms include decreased mRNA synthesis, increased mRNA degradation, or failure to process mRNA to an active translatable form.

Translatable mRNAs for several other proteins (including the 95,000- and 78,000-molecular-weight membrane proteins) were observed to increase in transformed cells. Recent experiments in this laboratory (19, 20) indicate that the induction of GRP-95 and GRP-78 is in large part secondary to the enhanced rate of glucose utilization of transformed cells that often depletes glucose from their growth medium. Neither protein is induced substantially in transformed cells that have been fed additional glucose to prevent glucose starvation; furthermore, they are induced in normal cells that have been starved for glucose (19, 20).

Three proteins that showed an increase when the RNAs were derived from transformed cells appeared to be synthesized independently of glucose metabolism. One of these has a molecular weight of 200,000 and immunoprecipitates specifically with affinity-purified anti-myosin (data not shown). A transformation-induced increase in translatable myosin mRNA appears to conflict with reports that the amount of myosin is not increased in transformed fibroblasts (34); this point is currently under investigation. The other translation products that increase have molecular weights of 87,000 and 72,000; current efforts are directed at establishing their nature.

In summary, our results show that the decrease in CSP and in collagen on transformation of CEF by Rous sarcoma virus is reflected by a decrease in functional mRNA for these proteins; in addition, we find quantitative changes in translatable mRNA for several other proteins. Thus, some of the major cellular changes induced by oncogenic viruses could be due to changes in the activity of specific cellular genes.

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