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Communication

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Declining Procollagen mRNA Sequences in Chick Embryo Fibroblasts Infected with Rous Sarcoma Virus

CORRELATION WITH PROCOLLAGEN SYNTHESIS*

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SUMMARY

Chick cells infected with Rous sarcoma virus are characterized by a wide variety of changes known collectively as transformation. Among these are decreases in the level of procollagen biosynthesis and in the level of procollagen mRNA. In this communication, we examine the time course of the decrease in procollagen biosynthesis, as measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and collagenase assay, and compare it with the decrease in procollagen mRNA sequences measured by hybridization to a complementary DNA. Procollagen biosynthesis and procollagen mRNA sequences decrease simultaneously after infection. Even the initial decrease in procollagen biosynthesis, therefore, is due to a decline in the level of procollagen mRNA.

The study of cellular changes induced by viral infection may elucidate cellular mechanisms in viral replication and in the normal regulation of host cell macromolecular synthesis. In eukaryotes, viral infection may or may not be followed by production of virus, and if production does ensue, it may be passive or lytic in nature. Thus, the extent to which host function is disrupted is dependent on the nature of the infection. For example, herpes simplex virus causes degradation of poly(A)-containing RNA within 4 h in lytically infected Friend leukemia cells (1). In the case of polio virus, host mRNA, although retained, is no longer translated because of changes in the host's translation machinery (2). On the other hand, Rous sarcoma virus induces a variety of changes in chick embryo cells, collectively known as transformation (3), and utilizes a relatively small percentage of host cell synthetic machinery for its replication (4).

A number of specific host functions, as well as the distribution of various proteins and mRNA sequences, are altered following transformation by RSV[1] (5); these include sugar transport (6), globin (7), sulfated proteoglycan (8), adenosine deaminase (9), fibronectin (10–12), and collagen (10, 13–18). However, relatively little is known about the mechanism or timing of these changes. In the case of procollagen, it has been shown that both synthesis of the protein and the procollagen mRNA level, when monitored by *in vitro* translation or hybridization to procollagen cDNA, are reduced in transformed cells (10, 17, 18).

In this study, we have measured the kinetics of the decrease in procollagen biosynthesis and in procollagen mRNA sequences during the first 84 h after infection of chick embryo fibroblasts with RSV. Specifically, we were interested in whether this initial reduction in procollagen synthesis could be attributed to events which affect the concentration of the message or to factors altering the activity of this mRNA. Our results indicate that procollagen synthesis is decreasing by 24 h after infection and that the time course of the decline in procollagen mRNA sequences and procollagen synthesis is similar.

EXPERIMENTAL PROCEDURES

Purification of Procollagen mRNA—Procollagen mRNA was purified from chick embryo tendons essentially as described by Rowe *et al.* (17). Total RNA was obtained by Proteinase K digestion in SDS followed by phenol/chloroform extraction. Total RNA was enriched for poly(A)-containing RNA by chromatography over oligo(dT)-cellulose. This RNA was further enriched for procollagen mRNA by size fractionation on sucrose gradients as described (17). Yields and enrichment for procollagen mRNA were similar to those obtained previously.

Translation of Procollagen mRNA—Translation was performed in a staphylococcal nuclease-treated rabbit reticulocyte lysate which had been chromatographed over Sephadex G-50. Conditions of translation were those optimized for procollagen synthesis by Rowe *et al.* (17). Products of *in vitro* translation were assayed by electrophoresis with procollagen standards in SDS-polyacrylamide gel electrophoresis as described by Laemmli (19), and observed by fluorography (20). Sensitivity to bacterial collagenase (Form III, Advance Biofactures) was determined by digestion as described by Peterkofsky and Diegelmann (21). The collagenase was shown to be free of nonspecific proteolytic activity.

Synthesis of [^{3}H]cDNA—The mRNA template was selected from sucrose gradient fractions containing proα1(I) and proα2 mRNA activities. cDNA was synthesized as described by Rowe *et al.* (17), and further purified by back-hybridization to polysomal RNA, enriched for procollagen mRNA sequences by immunoprecipitation with procollagen antibodies as described by Lee *et al.* (22). Fifty micrograms of immunoprecipitated polysomal RNA was hybridized to 100,000 cpm of procollagen cDNA for 14 h at 68°C using reaction conditions described by McKnight and Schimke (23). The hybridized cDNA was then chromatographed on hydroxyapatite followed by Sephadex G-75 chromatography to remove single-stranded cDNA and salt, respectively. About 40% of the counts per min in cDNA was recovered after back-hybridization to immunoprecipitated polysomal RNA. Double-stranded cDNA-RNA hybrids were base-treated in 0.3 N NaOH overnight to remove RNA. This purification increased saturation from about 30% to almost 60%.

Hybridization of cDNA—Hybridization reactions and quantitation of hybrids using S1 nuclease were carried out essentially as described (23).

Fibroblast Culture Conditions—Tendon fibroblasts were explanted from 17-day chick cells not susceptible to infection by sarcoma virus subgroup E (H and N Farms, Redmond, Wash.) according to the method of Dehm and Prockop (24). Cells were maintained in F-12 medium (GIBCO), 5% calf serum, and 10% tryptose/P_i broth in an atmosphere of 5% CO_2 at 39°C.

Kinetics of Infection—Cells were trypsinized and plated at a density of 1×10^6 cells/60 mm plastic tissue culture dish (Falcon). To one set of cells, an additional milliliter of medium was added, containing 1×10^6 focus forming units of Prague C strain of RSV and 10 μg of polybrene (both obtained as gifts from Robert Eisenman, Fred

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[1] The abbreviations used are: RSV, Rous sarcoma virus; SDS, sodium dodecyl sulfate; ts, temperature-sensitive.

Hutchinson Cancer Research Center, Seattle, Wash.). Control dishes received an additional milliliter of medium supplemented with polybrene alone. Medium was changed at 24 and 48 h. Cells were observed every 12 h after infection. At the times indicated, one infected and one control plate were washed three times with Dulbecco's modified Eagle's medium, preincubated in Dulbecco's modified Eagle's medium, supplemented with 50 μg/ml of ascorbate for 1 h, and then pulsed for 30 min with 2 ml of the latter medium supplemented with 25 μCi/ml of [2,3-^{3}H]proline. At the end of the pulse, each cell layer was divided into equal parts; half was dissolved in 0.5 M ammonium hydroxide and processed for protein determinations (25), collagenase digestion (21), and SDS-polyacrylamide gel electrophoresis (19), and half was taken up in SET buffer (1% SDS, 5 mM EDTA, 10 mM Tris, pH 7.5) containing 50 μg/ml of Proteinase K in SDS and extracted in phenol/chloroform as described above.

Cell layers dissolved in 0.5 M ammonium hydroxide were precipitated with trichloroacetic acid for collagenase assay as described by Peterkofsky and Diegelmann (21). The supernatants of this precipitation were counted to determine the radioactivity in proline pools in normal and transformed cells. Trichloroacetic acid-soluble pools of proline, expressed per μg of protein, in transformed cells were 1.4 times those in normal cells by 84 h. This could reflect larger pools of proline or higher specific activity of proline pools in transformed cells, or both. If the specific activity of the proline pools is not affected by transformation, then changes in collagenase-sensitive counts per min in proline, per μg of protein, would reflect absolute differences in collagen synthesized in normal and transformed cells. If, on the other hand, the specific activity of proline pools is greater in transformed cells, then measurement of collagenase-sensitive proline, expressed per μg of protein, may actually inflate the apparent level of collagen made by transformed cells, relative to normal cells. Data expressed as the percentage of total counts per min which is collagenase-sensitive are, of course, unaffected by differences between the specific activity of proline pools in normal and transformed cells.

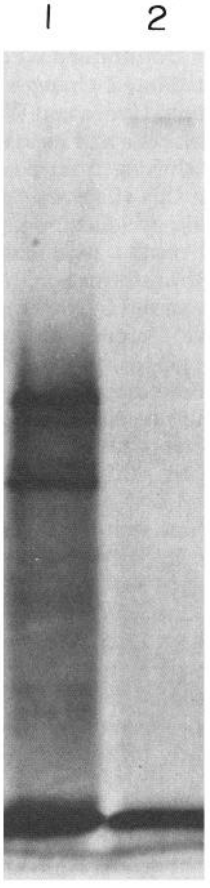

FIG. 1. SDS-polyacrylamide gel electrophoresis of translation products directed by procollagen mRNA in the reticulocyte lysate system. Poly(A)-enriched RNA was fractionated on sucrose gradients as described under "Experimental Procedures" and fractions containing mRNA activity for proα1(I) and proα2 chains were used to prime translations. Aliquots of the translation mixture, labeled with [^{3}H]-proline, were incubated minus (*Slot 1*) and plus (*Slot 2*) bacterial collagenase for 45 min at 37°C. Samples were electrophoresed directly on 5% SDS-polyacrylamide gels.

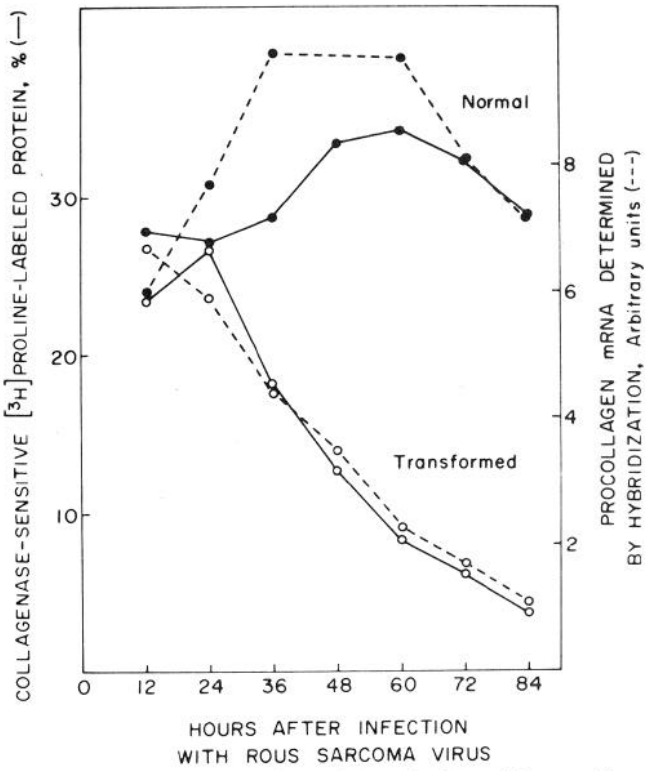

FIG. 2. Changes in procollagen biosynthesis and in procollagen mRNA sequences following transformation of chick embryo fibroblasts with RSV. Cells were labeled with [2,3-^{3}H]proline for 12, 24, 36, 48, 60, 72, and 84 h and processed as described under "Experimental Procedures." Hybridization values were normalized using a standard curve generated by hybridization with tendon mRNA. ——, collagenase-sensitive protein in normal (●——●) and transformed (○——○) cells; - - -, hybridization levels in normal (●- - -●) and transformed (○- - -○) cells.

RESULTS

Purification of Procollagen mRNA and Synthesis of Procollagen cDNA—Translation of sucrose gradient-fractionated chick tendon poly(A)-containing RNA revealed a broad region of procollagen mRNA activity spanning the marker 28 S rRNA peak (data not shown). Electrophoresis of translation products on SDS-polyacrylamide gels (Fig. 1, *Slot 1*) showed that the mRNA activity in this region of the gradient stimulated the synthesis of proteins migrating in the region of chick proα1(I) and proα2 markers. The existence of doublets in each of these positions was variable and could have been due to partial proteolysis of the procollagens by the lysate itself. Fig. 1 demonstrates that these bands were sensitive to bacterial collagenase; the products obtained by translation of oviduct mRNA were not (data not shown). Interestingly, some mRNA template activity for proα2 migrated perceptibly earlier in the sucrose gradient than did proα1(I) activity (data not shown). Procollagen cDNA was synthesized from pooled fractions of the gradient containing mRNA activity for both proα2 and proα1(I) chains.

Kinetics of the Decrease in Procollagen Synthesis Following Infection with RSV—The time course of the decline in procollagen synthesis in chick embryo tendon fibroblasts following infection with RSV is shown in Fig. 2. Procollagen synthesis, as indicated by collagenase-sensitive proline-labeled protein in the cell layer, declined starting 24 h after infection

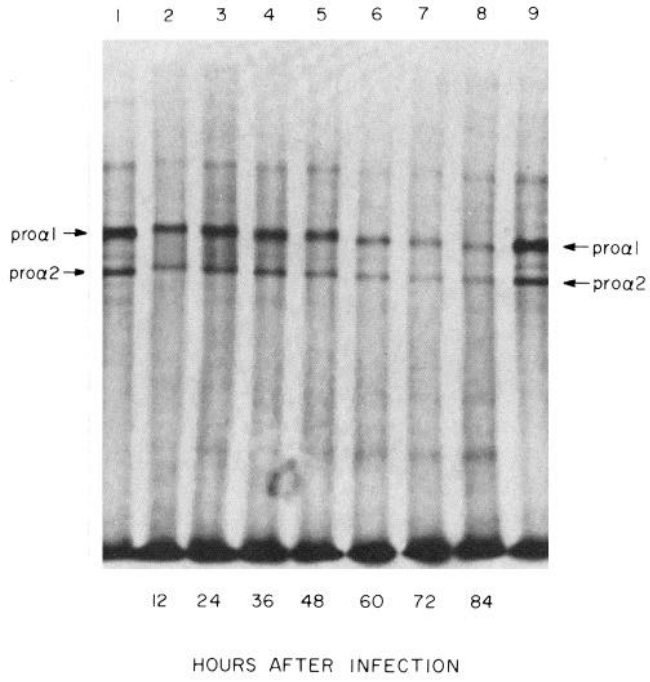

FIG. 3. SDS-slab gel electrophoresis of [2,3-^{3}H]proline-labeled protein in chick embryo fibroblasts following infection with RSV. Proteins were precipitated with 10% trichloroacetic acid, washed with ethanol and ethanol/ether, and dissolved in sample buffer containing 50 mM dithiothreitol. Equal amounts of radioactivity were loaded in each slot. *Horizontal arrows* indicate the positions of migration of chick proα1(I) and proα2 chains. *Slots 1* and *9*, uninfected cells after 84 h; *Slots 2* to *8*, cells at 12, 24, 36, 48, 60, 72, and 84 h after infection.

and was still declining 84 h after infection. In contrast, procollagen synthesis in cells not infected with RSV actually increased somewhat over this time course, perhaps as a response to rapid proliferation upon plating. If the data are expressed as collagenase-sensitive counts per min per mg of cellular protein, instead of as a percentage of total counts per min, a similar decrease in infected cells is observed. This suggests that the decrease is an absolute one and is not only a change in the percentage of total protein synthesis devoted to collagen.

Examination of procollagen mRNA levels by hybridization to procollagen cDNA revealed a decline in procollagen sequences following RSV infection. A decrease was apparent by 24 h after infection and the decline continued in parallel with the change in procollagen synthesis up to 84 h after infection. In contrast, in uninfected cells, the level of procollagen mRNA levels increased significantly until 36 h, then reached a plateau and returned to initial levels by 84 h. It thus appears that the decline upon infection in procollagen biosynthesis can be accounted for by a corresponding decrease in procollagen mRNA levels.

These results are corroborated by SDS-polyacrylamide gel electrophoresis analysis, as shown in Fig. 3. In this experiment, equal counts of proline-labeled cell layers were electrophoresed on 5% polyacrylamide gels. The decrease in the density of the bands co-migrating with proα1(I) and proα2 chains over the time course is apparent. Several new proteins, which may be transformation-related, appear late in the time course of infection. Densitometric tracing of the fluorogram revealed that, at 48 h after infection, radioactivity in the two procollagen bands of the transformed cells (Fig. 3, *Slot 5*) was 43% of that in the control cells (Fig. 3, *Slot 1*). By 84 h, transformed cell procollagen bands (Fig. 3, *Slot 8*) had decreased to 14% of the control value. These data are in good agreement with those obtained by collagenase digestion.

DISCUSSION

Clear definition of the transition from normal to transformed states in cells infected by RSV will help to characterize the points at which transformational control is exerted. Our work has demonstrated that the reduction in procollagen synthesis observed in RSV-transformed cells is mediated by a decrease in the level of procollagen mRNA sequences soon after infection. Other workers have shown that induction and maintenance of transformed characteristics in chick cells, infected by RSV, depends on the *src* gene product (26), a protein kinase (27). There is also evidence from experiments on cells infected with RSV, which was ts in the transformation function, that the *src* protein acts at both nuclear and cytoplasmic sites (28).

Since fibronectin, like procollagen, is a cell surface-associated and extracellular connective tissue protein which is reduced in RSV-transformed chick cells (10–12), it is informative to briefly compare what is known about the mechanism of its reduction with the results reported here. In fully transformed cells, Olden and Yamada (11) have shown that changes in both biosynthesis and extracellular turnover contribute to the decreased level of fibronectin. At least part of the decrease in biosynthesis is attributable to a decrease in the level of translatable fibronectin mRNA, as assayed *in vitro* (10). Experiments with chick cells transformed with RSV ts for transformation have also elucidated changes in post-translational regulation of this protein. Hynes and Wyke (12) found that ts RSV-transformed chick cells had increased cell-surface fibronectin within 4 h of shifting from permissive to restrictive temperatures and that this effect was insensitive to an inhibitor of protein synthesis, cycloheximide. Beug *et al.* (28) have obtained compatible results with chick fibroblasts transformed by RSV ts for transformation. In the latter work, cell-surface fibronectin measured by immunofluorescence was absent at the permissive temperature in both nucleated and enucleated cells, but reappeared upon shifting to the restrictive temperature. It therefore seems that the transformation-related reduction of fibronectin may involve an initial modulation of fibronectin levels which is independent of the change in the level of fibronectin mRNA.

The results presented in this communication are of interest since this is the first time that the reduced level of a cellular protein after infection by RSV could be correlated with the initial decrease in mRNA for that protein. Our experiments suggest that, in the case of procollagen, the regulation of procollagen mRNA is at least partially responsible for the initial decrease observed in procollagen biosynthesis. Since the experiments presented here have measured cell layer procollagen labeled in a 30-min pulse period, these measurements do not necessarily reflect changes in turnover of cell surface or extracellular protein, or both. It will be interesting to compare the results of additional studies relating to the regulation of fibronectin and procollagen in transformed cells.

The decrease in procollagen mRNA levels shown here could result from decreased synthesis, increased degradation, or a combination of these factors. Experiments are underway to distinguish among these possibilities.

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