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Coordinate Transcriptional Regulation of Type I Procollagen Genes by Rous Sarcoma Virus*

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Chicken embryo fibroblasts infected with a strain of Rous sarcoma virus containing a temperature-sensitive mutation in the gene coding for pp60^{src}, a protein kinase, undergo changes in collagen synthesis within 4 h after a temperature shift. Cells shifted from the restrictive to the permissive temperature for transformation show decreasing levels of collagen synthesis and increasing levels of kinase activity; the reverse occurs when infected cells are shifted from the permissive to the restrictive temperature. Levels of type I procollagen mRNAs coding for proα1 and proα2 chains, measured by hybridization to nick-translated cloned α1 and α2 cDNA, decreased simultaneously soon after a reduction in temperature and reached a new steady state at about 50 h after the shift.

In order to test for regulation at the transcriptional level, nuclei were isolated from normal and Rous sarcoma virus-transformed chicken embryo fibroblasts and allowed to transcribe in the presence of [α-^{32}P]UTP. Procollagen mRNA sequences in newly synthesized and in total RNA from transformed cell preparations were both about 5-fold lower than the levels in normal cell preparations. We conclude that the coordinate decrease in procollagen mRNAs observed in Rous sarcoma virus-transformed chicken embryo fibroblasts is caused primarily by a decrease in the transcription of the type I procollagen genes, a decrease which is directly or indirectly mediated by the pp60^{src} protein kinase.

Chicken embryo fibroblasts infected with RSV[1] undergo a variety of changes in cellular biochemistry, including alterations in glycolipid structure (1), increased hyaluronate synthesis (2) and sugar transport (3), reduced cAMP levels (4), decreased amounts of surface fibronectin (5), and decreased collagen synthesis (6–8) and procollagen mRNA levels (9–13). If cells are infected with a mutant of the virus which has a *src* gene deletion, the virus integrates and replicates normally, but the biochemical changes associated with transformation do not occur (for a review, see Ref. 14).

It seems likely that a single viral gene which can produce such pleiotropic effects must code for a function which mimics a key element in cellular regulation. Investigators have found that the *src* gene codes for a phosphorylated polypeptide of M_r = 60,000, denoted pp60^{src} (15–17), which has an associated protein kinase activity (17–19). In addition, a structurally related protein kinase, termed pp60^{sarc}, has been found in uninfected vertebrate cells (20–23). pp60^{sarc} activity is present in normal cells at about 1/50 the level of pp60^{src} activity in RSV-transformed CEF (21, 23, 24). Because transformation specifically affects collagen synthesis, a major differentiated function of chicken embryo fibroblasts, it provides a system in which one can study the action of the pp60^{src} kinase and perhaps the nature of normal regulation as well.

In this paper we describe a series of experiments undertaken to elucidate the mechanism of the decrease in collagen synthesis observed in transformed cells. We were particularly interested in a more detailed study of the events that take place in CEF undergoing transformation, the increase in pp60^{src} kinase activity, the decrease in procollagen mRNA, and the decrease in collagen synthesis, and in determining whether the decrease in procollagen mRNA is correlated with a decrease in messenger RNA synthesis. Our results show that there is an inverse correlation between pp60^{src} kinase activity and the levels of type I mRNA and collagen synthesis and that the reduced mRNA levels can be accounted for by a reduction in the transcription of α1 and α2 procollagen genes.

EXPERIMENTAL PROCEDURES[2]

Viruses

The Prague A strain of RSV, PR-RSV-A, was a gift from Maxine Linial, Fred Hutchinson Cancer Research Center, Seattle, WA. RSV tsLA24 (25), a derivative of the Prague A strain which contains a temperature-sensitive mutation in the src gene, was also obtained from Dr. Linial. Infected cells are normal at the restrictive temperature of 41° C, but transformed at the permissive temperature, 35° C. This virus was subcloned and the four subclones producing the greatest temperature-dependent change in morphology were assayed for collagen synthesis at 35° C and 41° C. The clone which caused infected cells to have the lowest collagen synthesis (most transformed) at 35° C and the highest collagen synthesis (most normal) at 41° C was designated tsLA24-10 and was used in the studies described here.

Cell culture

Primary cells were obtained from chicken embryos which were chicken helper factor negative and resistant to sarcoma virus subgroup E (C/E) (H and N Farms, Redmond, WA). CEF cultures were started by dispersing the body wall of 11-day embryos by shaking at 37° C in a mixture of 0.05% trypsin in DMEM, containing penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml). CEF were maintained in F-10 medium supplemented with 5% fetal calf serum (Irvine Scientific), 10% tryptose phosphate broth, and penicillin as described above. Unless specified, cells were maintained at 39° C in a 5% CO_2 atmosphere.

Cells, used for collagenase assay and RNA determinations were harvested just prior to confluence (except for cultures late in the course of a temperature shift). Cultures were maintained for two or three passages before viral infection to reduce contamination with nonfibroblastic cell types. At the third or fourth passage, cells were infected with Prague A or tsLA24-10, at a multiplicity of infection of approximately one focus-forming unit/cell.

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[1] The abbreviations used are: RSV, Rous sarcoma virus; CEF, chick embryo fibroblasts; EDTA, ethylenediamine tetraacetic acid; DBM, diaminobenzyloxymethyl.

[2] The "Experimental Procedures" are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 80 M-2385, cite author(s), and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

Temperature shift of tsL24-10-infected CEF

Measurements of collagen synthesis, pp60^{src} activity, and procollagen mRNA levels were made at various times after a temperature shift. Cells were infected with tsL24-10 and maintained at 35° C or 41° C. After four days, morphological transformation of cultures maintained at 35° C was always complete. Cells were replated at subconfluent density without temperature shift and the medium was changed on the second day. On the third day, sets of cells were shifted from 41° C to 35° C or from 35° C to 41° C or maintained at 41° C or 35° C.

Immunoprecipitation of pp60^{src} kinase activity

pp60^{src} kinase activity was measured as the transfer of ^{32}P by immunoprecipitated pp60^{src} from [γ-^{32}P]ATP to the anti-pp60^{src} immunoglobulin gamma chain. Antiserum against pp60^{src} from tumor-bearing rabbits was a gift from Larry Rohrschneider. The preparation and properties of this serum have been described in detail (26). The antiserum we employed precipitated both viral pp60^{src} and the endogenous pp60^{src} protein from uninfected chicken cells (22). The preparation of cell extracts for assay of pp60^{src} kinase activity, and the assay itself, were performed as described by Collett and Erikson (19). Cell lysates were handled at 4° C. Immunoprecipitation was performed with 4 µl of antiserum and 100 µl of 100 mg/ml fixed Stephylococcus aureus; this procedure has been described in detail (27). All kinase reactions were incubated for 10 min at 30° C with 0.1 µM [γ-^{32}P]ATP (3000 Ci/mmole, Amersham). Following the reaction, immunoprecipitates were washed three times with buffer (19) and once with 0.01 M Tris, pH 7.5; electrophoresis sample buffer (28) was then added and immunoprecipitates were boiled for 2 min. Samples were centrifuged and the supernatants containing the phosphorylated immunoglobulin were recovered for gel electrophoresis.

Using this assay we determined that incorporation of ^{32}P increased linearly with increasing input of cellular extracts between 0 and 200 µg of cellular protein; all measurements were therefore performed within this range. Protein was determined by a dye-binding assay (29).

Polyacrylamide gel electrophoresis, autoradiography, and radioactivity in the phosphorylated heavy chain

Phosphorylated protein from the immunoprecipitates described above was fractionated on a 12.5% polyacrylamide gel (28). The gel was stained, destained, and dried onto Whatman 3 MM paper. Radioactivity was detected by exposure to Kodak X-Omat R film. Each portion of the gel containing phosphorylated immunoglobulin heavy chain was cut out and dissolved by incubation in a scintillation vial in 1 ml of 30° H_2O_2 for 1.5 h at 80° C. The vials were cooled, toluene-based scintillant was added and the radioactivity was counted.

Collagen synthesis

Collagen synthesis in the CEF cells was determined by collagenase assay described by Peterkofsky and Diegelmann (30). Cells were preincubated in Dulbecco's modified Eagle's medium (DMDM) supplemented with 50 µg/ml ascorbate and antibiotics for 1 hr and labeled at various times during a time course by incubation for 30 min in 20-25 µCi/ml of [2,3-^{3}H]proline in the above medium. Cell layers were washed three times with cold phosphate buffered saline, solubilized in 0.5 M ammonium hydroxide, precipitated by bringing the solution to 10% trichloroacetic acid, and further processed as described (30). Digests were carried out with Form III collagenase (Advanced Biofactures Corp.). No nonspecific proteolytic activity was detected when the collagenase was incubated with noncollagenous [^{3}H]tryptophan-labeled substrate under the conditions of the assay.

Recombinant DNA

E. coli HB101, containing the plasmid pBR322 was a gift from Stanley Falkow, University of Washington, Seattle, WA. The α1 and α2 procollagen cDNA clones, pCg54 and pCg45, respectively, used in these studies were gifts from Helga Boedtker, Harvard University, Boston, MA, and have been described previously (31,32). Both cDNAs were introduced into the unique HindIII site of pBR322 using synthetic HindIII linkers. The plasmids were carried in the EK1 host HB101. The clones have been analyzed and identified by restriction mapping, sequencing, and translation of hybrid-selected RNA. PCg54 carries an 1100 base pair sequence coding for a portion of the α1 procollagen chain. PCg45 carries a 2500 base pair sequence coding for a portion of the α2 procollagen chain. Recombinant cultures were maintained under P2 containment according to the National Institutes of Health Guidelines as directed by the University of Washington Institutional Biohazards Committee.

Plasmid isolation was performed as described by Tanaka and Weisblum (33) except that plasmids were not purified by equilibrium sedimentation. The final plasmid preparation was electrophoresed on 0.7% agarose (34) to check for host DNA contamination. Each plasmid preparation was also identified by restriction digestion and gel electrophoresis (34).

Procollagen-specific fragments were prepared by restricting the pCg54(α1) plasmid with HindIII and KpnI (32) according to conditions specified by the supplier (BRL), and pCg45(α2) with HindIII alone (31). Procollagen-specific fragments were separated from the remainder of the plasmids by electrophoresis in 0.7% agarose gels and were recovered from a slice of the gel by electroelution. Contaminants coelectroeluting from the agarose, which potentially interfere with subsequent enzymatic treatment of the fragment, were removed by chromatography over a small DEAE-cellulose column equilibrated in gel electrophoresis buffer. The column was washed further with this buffer and the DNA was eluted with buffer containing 1 M NaCl. The recovered fragment was precipitated overnight with two volumes of ethanol at -20° C and centrifuged at 35K in an SW40 rotor (Beckman) at 4° C for 45 min.

Nick translation

Nick translation was carried out essentially as described by Thomashow et al. (35). The reaction mixture was 20 µM in dTTP, dATP, and dGTP. 50-100 µCi [α-^{32}P]dCTP (greater than 300 Ci/mmol) per µg DNA gave specific activities of up to 1 x 10^{8} cpm/µg DNA.

RNA isolation

RNA was purified as described by Rowe et al. (11). Total nucleic acid was obtained by proteinase K digestion in SDS followed by phenol/chloroform extraction and ethanol precipitation. Nucleic acid was dissolved in water and precipitated with two volumes of 3 M sodium acetate, 5 mM EDTA, pH 6.0, at 4° C for several hr. Precipitated RNA was washed once with the above salt buffer and twice with 60% ethanol, 0.1 M NaCl. The pellet was resuspended in water or 0.1% SDS.

Linkage of RNA and DNA to cellulose

Aminobenzyloxymethyl cellulose (Miles) was converted to cellulose powder and diazotized to form diaminobenzyloxymethyl (DBM) cellulose as described by Noyes and Stark (36). After diazotization, the DBM cellulose was washed once with cold water and twice with cold 0.2 M sodium phosphate, pH 5.5. The cellulose was then suspended at 10-50 mg/ml in 20 mM sodium phosphate, pH 5.5, for linkage to RNA. RNA, dissolved in 20 mM sodium phosphate, pH 5.5, was then added to the cellulose at a concentration of 10 µg per mg. The linkage reaction proceeded with shaking at 4° C for 24-48 hr. RNA-cellulose was then incubated at 42° C for 4-12 hr in 50% deionized formamide, 0.75 M NaCl, 75 mM sodium citrate, 0.2% each of bovine serum albumin, ficoll, and polyvinylpyrrolidone, 100 µg denatured E. coli DNA/ml, and 1% glycine (37). This step was important in reducing background, probably by eliminating unreacted diazo groups. RNA linkage to cellulose as monitored by retention of [^{3}H]RNA and A_{260} was greater than 95%, up to concentrations of 10 µg/mg cellulose.

DNA linkage was carried out in the same manner except that plasmid DNA was heated in a boiling water bath for 8 min to nick and denature the DNA, and the DBM cellulose was washed once with 20 mM sodium phosphate, pH 5.5, to reduce the salt concentration.

Hybridization assay of total cellular RNA

Hybridizations were carried out between a nick-translated DNA fragment coding for α1 and α2 sequences (see above) and RNA linked covalently to cellulose. Hybridization reactions were performed in DNA excess in 80% deionized formamide, 0.4 M NaCl, 0.01 M PIPES, pH 6.4 (38), containing 100 µg denatured E. coli DNA/ml. Hybridization assays contained 2-7 x 10^{5} cpm of probe and 0.01-10 µg RNA in 200 µl. Reactions were covered with paraffin oil and shaken in round-bottom plastic tubes at 50° C for 24-72 hr. Under these conditions up to 90% of the cDNA could be driven into DNA-RNA hybrids. Following the hybridization reaction, the cellulose was washed three times in warm 0.60 M NaCl, 0.06 M sodium citrate containing 0.1% SDS, and 1 mM EDTA to remove most of the nonhybridized label. Background was further reduced by three additional centrifugations alternated with shaking in wash buffer for 30 min each at 60° C. Background, which was proportional to the quantity of cellulose, was determined by carrying a series of concentrations of blank cellulose through a mock hybridization and wash, and was subtracted from the raw hybridization cpm.

Hybridization conditions were determined by a number of factors. A temperature of 50° C and addition of 100 µg/ml E. coli DNA favored specific hybridization. The formamide, salt and buffer conditions favored RNA-DNA hybridization and minimized degradation (38). Nick translation results in a significant percentage of snapback sequences (39). We observed 7-10% snapback sequences with circular plasmid substrate and as much as 25-30% snapback sequences with linear substrates such as the procollagen-specific restriction fragments used here. Since this would create an unacceptable background in solution hybridization, and greatly reduce the sensitivity of measurements which could be made, we linked the RNA to cellulose so that only the DNA probe hybridized to RNA would be recovered. Cellulose powder was used in order to optimize contact between the DNA probe and matrix-bound RNA, and because on a weight basis the powder has a far higher capacity to bind RNA than does filter paper. Customarily, RNA was linked to cellulose in a batch reaction and then assayed at concentrations from 0.01-10 µg of RNA per reaction. Although hybridization is readily observed below 1 µg of RNA per hybridization reaction, we found that higher amounts of RNA gave greater sensitivity.

Nuclear transcription, isolation of RNA, and hybridization

Nuclei were isolated essentially as described by Mulvihill and Palmiter (40) from CEF cells at early confluence and were stored until use in liquid nitrogen. Nuclear transcriptions were carried out as described by McKnight and Palmiter (41). Each 100 µl of reaction mix contained 25 to 70 µg of nuclear DNA and 100 µCi [α-^{32}P]UTP (greater than 300 Ci/mmol). Transcription reactions were incubated for 10 to 60 min at 26° C. The KCl concentration specified by McKnight and Palmiter for oviduct nuclei was optimal for total incorporation in CEF nuclei as well. Nuclear incorporation was 1.5-5 x 10^{5} cpm/µg DNA. RNA was isolated as described (41). The RNase-free DNase used in the isolation was a gift from Richard Palmiter, University of Washington, Seattle, WA. The procollagen cDNA plasmids, pCg54 and pCg45, were baked onto 7 mm diameter circles of nitrocellulose paper (Beckman) at 1 and 1.2 µg/filter, respectively, according to the method of Gillespie (42). Hybridizations were in DNA excess. The conditions of hybridization were as described [0.5 M NaCl, 50 mM PIPES, pH 7.0, 33% deionized formamide, 0.4% SDS, 2 mM EDTA; McKnight and Palmiter (41)], but volumes varied from 40-60 µl/assay. Three filters were included in each hybridization: a pCg54(α1), pCg45(α2), and pBR322 or blank filter. Reactions were incubated at 45° C for three days. Nonspecific label was removed from the filters by washing and RNase A and T1 digestion and filters were counted as described (41). Backgrounds were between 5 and 10 ppm and were subtracted from α1 and α2 filter hybridizations.

RESULTS

Immunoprecipitation of Protein Kinase Activity from Uninfected and Infected Cells—A pp60^{src}-associated protein kinase activity has been detected in both RSV-infected and uninfected vertebrate cells (20–22). This kinase activity has been measured by precipitation of the enzyme with antiserum against pp60^{src} from tumor-bearing rabbits. The activity is about 50- to 100-fold greater in transformed than in normal cells (21, 23, 24). It is also temperature-sensitive in extracts from cells infected with a virus possessing a temperature-sensitive mutation in the *src* gene (17, 19, 43–45). The phosphorylation of the IgG by this kinase may reflect the *in vivo* activity of the pp60^{src} protein, although the natural substrate(s) in the cell is unknown.

Nonimmune serum, reacted with infected and uninfected CEF extracts, did not result in phosphorylation of the IgG heavy chain. However, the heavy chain of IgG from anti-pp60^{src} serum was phosphorylated by extracts from uninfected cells and to a much larger extent by extracts from infected cells (data not shown). Quantitation of the cpm incorporated into the heavy chain revealed a 60-fold greater phosphoryla-

tion of the IgG by infected than uninfected cell extracts. As a control, antiserum incubated with *Staphylococcus aureus* cells, but without cell extract, showed no ability to transfer ^{32}P from [γ-^{32}P]ATP to IgG.

Measurements of Kinase Activity and Collagen Synthesis in Normal and Transformed Cells after Shifts to Temperatures Permissive and Restrictive for Transformation—We have measured levels of procollagen mRNAs early after infection of CEF with RSV and have shown that these mRNA levels begin to decline about 24 h after infection (13). In order to eliminate the time required for provirus synthesis, integration, and gene expression (see Ref. 46 for a review) and to observe directly the effects of transformation on collagen synthesis and mRNA levels, we have infected CEF with a mutant temperature-sensitive (ts) for transformation, tsLA24 (25). We then shifted the cells to temperatures both permissive and restrictive for transformation and measured levels of collagen protein and mRNA and pp60^{src} kinase activity over a period of 2–3 days.

The amount of immunoprecipitable kinase activity in uninfected and infected cells during the course of a temperature shift was determined. Since the antiserum reacts with endogenous pp60^{sarc}, as well as with viral pp60^{src}, the activity of uninfected cells was subtracted from that of infected cells. Normal and transformed cells were at approximately the same density throughout the time course, as determined by protein content/plate, so no corrections were necessary for changes in activity of pp60^{sarc} with different cell densities. The normalized results are shown in relation to changes in collagen synthesis over the time course (Fig. 1, *A* and *B*). Pp60^{src} kinase activity increased abruptly during the shift to the permissive temperature (Fig. 1*A*) and decreased abruptly during the shift to the restrictive temperature (Fig. 1*B*). The 3- to 6-fold range of kinase activity between restrictive and permissive conditions has been observed by other investigators (44, 47). The difference is less than that observed between wild type RSV-transformed CEF and normal CEF, probably because the temperature-sensitive transforming protein is less active than the wild type protein at the permissive temperature and has residual activity at the restrictive temperature.

Collagen synthesis was measured by collagenase assay in tsLA24-10-infected cells shifted from 41–35 °C (Fig. 1*A*) and from 35–41 °C (Fig. 1*B*). Infected cells shifted from 41–35 °C showed a decrease in collagen synthesis within 4 h after the shift in temperature. Levels of collagen synthesis continued to decrease for almost 72 h and eventually reached a level about one-quarter of that synthesized at 41 °C. The increase in collagen synthesis over the time course of cells shifted from 35–41 °C (Fig. 1*B*) was almost a reverse of the experiment shown in Fig. 1*A*. In the shift from the permissive to the restrictive temperature the increase in collagen synthesis is apparent by 4 h and it continued to increase to 72 h. At this time, the cells synthesized slightly more than 4 times the level of collagen synthesized at 35 °C. These changes were not observed in uninfected cells subjected to such temperature shifts. The reason for the continued increase in collagen synthesis beyond the level shown by tsLA24-10-infected cells at 41 °C is not clear, but it is probably a function of the density of the cells at the time of the temperature shift.

The change in anti-pp60^{src}-precipitable kinase activity levels was not maximal before the change in levels of collagen synthesis was initiated. Hence, it appears that there does not have to be full expression or complete loss of pp60^{src} kinase activity in order to induce changes in collagen biosynthesis.

Determination of Procollagen mRNA Content in Normal and Transformed Cells—In order to determine whether changes in the levels of collagen synthesis might be under

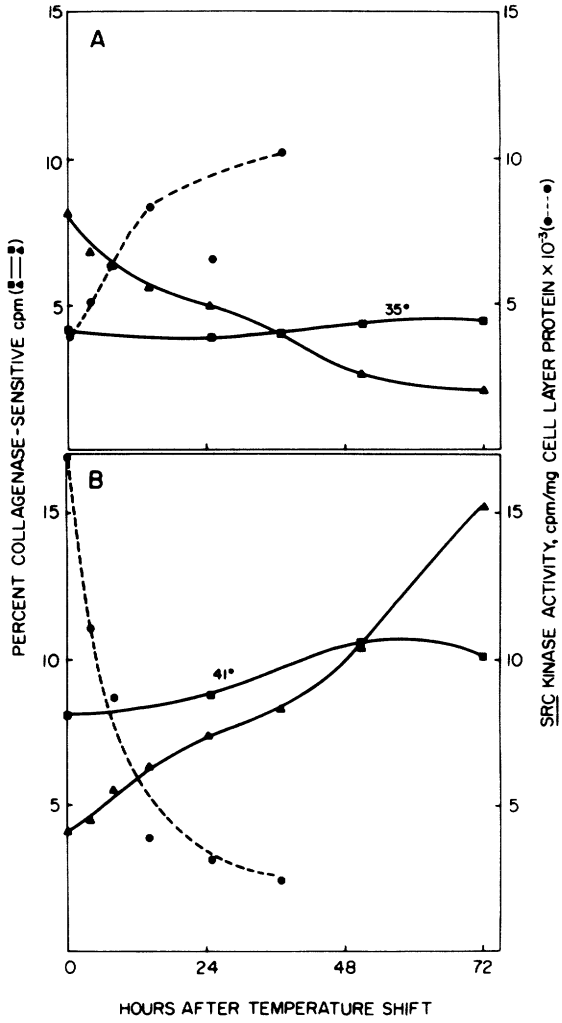

FIG. 1. **Effects of shifts to permissive and restrictive temperatures on pp60^{src} kinase activity and collagen synthesis in tsLA24-10-infected CEF.** CEF were infected at 35 °C with tsLA24-10 and after 2 days were passaged and maintained at 41 or 35 °C. After 4 days, uninfected and infected cells at both temperatures were plated at 500,000 cells/60-mm plate. Cells were temperature-shifted on the second day after replating. At $t = 0$, dishes were shifted from 41–35 °C (*Panel A*) or from 35–41 °C (*Panel B*). At various times after the temperature shift, plates were quick-frozen at −70 °C and later assayed for pp60^{src} kinase activity (●- - -●) by γ chain phosphorylation and for collagen synthesis (▲——▲) by collagenase assay. There was no loss of kinase activity in cells stored at −70 °C for up to 1 month. Ts-LA24-10-infected cultures were maintained at the original temperature in each case and assayed for collagen synthesis (■——■).

transcriptional control we measured α1 and α2 procollagen mRNA levels in normal and transformed cells by hybridization with nick-translated procollagen-specific fragments of pCg54 and pCg45, respectively. Fig. 2 shows that the extent of hybridization was directly proportional to RNA up to 20 μg of total RNA/assay. The extent of hybridization was also dependent on DNA input and time. RNA from transformed cells contained 7.6-fold less α1 procollagen mRNA and 4.3-fold less α2 procollagen mRNA than RNA from normal cells.

Since the α1 and α2 collagen chains show considerable homology (for a review, see Ref. 48), it was necessary to show that the two mRNAs could be measured independently in our assay. We prepared nick-translated fragments from pCg54 and pCg45, representing the α1 and α2 sequences, respectively, and hybridized 500,000 cpm of each to whole pCg54 and pCg45 plasmids attached to cellulose (10 μg of pCg54/mg and 12.2

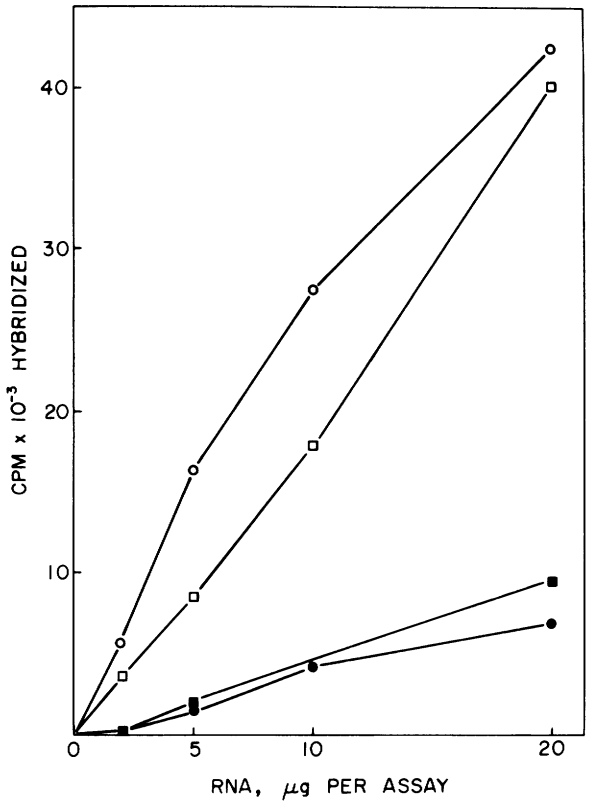

FIG. 2. **Levels of α1 and α2 procollagen mRNAs in normal and transformed CEF.** RNA was isolated as described from normal (○, □) and RSV-transformed (●, ■) CEF. The RNA was covalently linked to DBM-cellulose at a concentration of 10 μg of RNA/mg of cellulose and hybridized to nick-translated restriction fragments from pCg54 (◑——◑) and pCg45 (◧——◧). The molar input of α1 (pCg54) and α2 (pCg45) probes was identical; 2×10^5 cpm/assay of the α1 probe and 7.5×10^5 cpm/assay of the α2 probe were used.

μg of pCg45/mg). Low cross-reactivity between the probes was, in fact, observed (Table I). The α1 probe hybridized to pCg45 at 1.3% of the level to which it hybridized to its complementary plasmid, pCg54. The α2 probe hybridized to pCg54 at 8.1% of the level at which it hybridized to its complementary plasmid, pCg45. Since some contamination of the nick-translated fragments with pBR322 sequences is likely, these estimates provide upper limits of the extent of cross-hybridization in the actual RNA assays.

Temperature Dependence of Procollagen mRNA Levels in tsLA24-10-infected Cells—α1 and α2 procollagen mRNA levels in tsLA24-10-infected CEF shifted from 41–35 °C were measured by hybridization assay. Fig. 3 shows the kinetics of the change in relative concentration of procollagen mRNA in RNA isolated from cells before the temperature change and up to 50 h after the shift to 35 °C. Even at early times after the shift to the permissive temperature, α1 and α2 procollagen mRNA levels had dropped from values observed at 41 °C. The decrease is most dramatic between 5 and 10 h after temperature reduction but there was some variability in timing. By 50 h after the temperature shift, proα1 and proα2 mRNA levels had decreased to 26 and 32% of the original levels, respectively. In contrast, uninfected cells subjected to a similar shift in temperature showed no reduction in α1 and α2 procollagen mRNA levels as measured by hybridization (data not shown).

Regulation of Transcription of the Type I Procollagen Genes by RSV—To ascertain whether the decrease in collagen synthesis and procollagen mRNA levels is due to a decrease in the rate of transcription of the type I procollagen genes, we isolated nuclei from normal and transformed CEF and allowed

TABLE I

Cross-reactivity of α1 and α2 plasmid probes

DNA linked to cellulose	Nick-translated probe[a]	Amount recovered[b]	% of input
		cpm	
None	α1[c]	4813 ± 314	0.9
	α2[d]	967 ± 174	0.1
pCg54	α1	197,138 ± 2747	39.4
	α2	8759 ± 32	1.7
pCg45	α1	2679 ± 305	0.5
	α2	107,413 ± 6356	21.4

[a] Input was 5×10^5 cpm/reaction.
[b] Background was determined in hybridizations with *Escherichia coli* DNA linked to cellulose and subtracted.
[c] The α1 probe was a 1100-base-pair proα1 fragment from pCg54.
[d] The α2 probe was a 2500-base-pair proα2 fragment from pCg45.

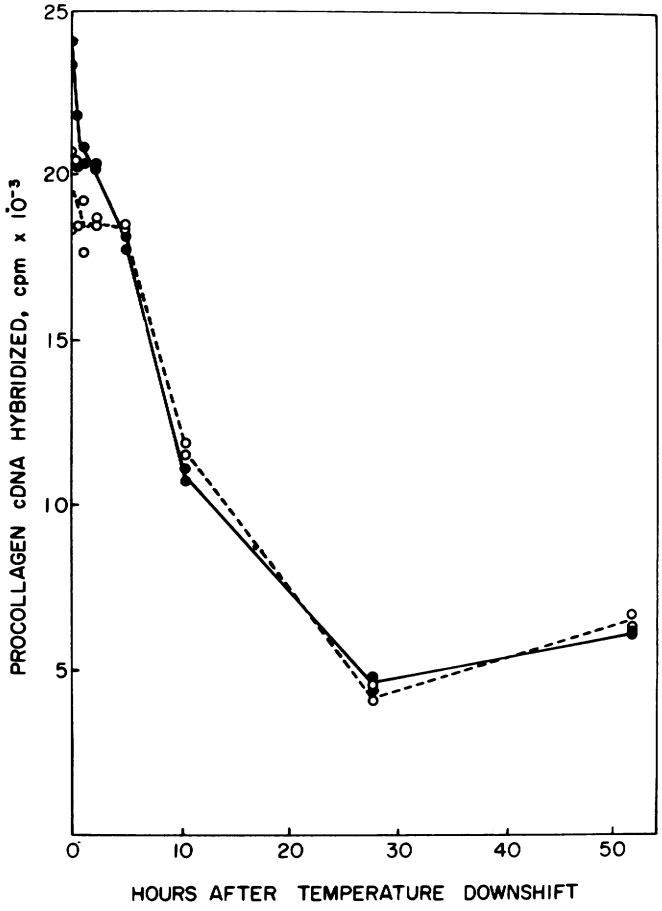

FIG. 3. **Effect of shift to the permissive temperature on α1 and α2 procollagen mRNA levels in tsLA24-10-infected CEF.** tsLA24-10-infected CEF were infected and maintained at 41 °C as described under "Experimental Procedures." Control infected cells were also kept at 35 °C to monitor the course of the infection. After 4 days cells were replated at 2×10^6 cells/135-mm dish. Cells were temperature-shifted on the second day after replating. RNA was isolated from tsLA24-10-infected CEF at 0, 0.5, 1, 2, 5, 10, 27, and 50 h after shifting from the restrictive temperature (41 °C) to the permissive temperature (35 °C) and attached to cellulose at a concentration of 10 μg/mg. α1 (●——●) and α2 (○- - -○) mRNA levels were assayed by hybridization to α1 and α2 nick-translated probes for 2 days; 5×10^5 cpm were used in each assay.

them to transcribe in the presence of [α-^{32}P]UTP; transcripts were isolated and hybridized to α1 and α2 plasmids bound to filters. Fig. 4 shows that hybridization of α1 and α2 RNA sequences synthesized *in vitro* by nuclei from normal CEF increased with input up to 3×10^6 cpm, indicating that plasmid DNA is in excess.

In another experiment, we compared the relative level of procollagen mRNA transcription and steady state mRNA levels of normal and transformed cells (Fig. 5). The results of hybridization of nick-translated α1 and α2 cDNA fragments to RNA bound to cellulose are shown in *Panel A* (Fig. 5). The α1 and α2 mRNA in transformed cells are present at 13 and 24% of the level observed in normal cells, respectively. These levels correlate well with the level of collagen synthesis observed in transformed cells, about one-seventh of the level in normal cells. Nuclei from duplicate plates were isolated and transcribed *in vitro*. Total incorporation was similar for normal and transformed nuclei. On the basis of ppm of input hybridized, the relative rate of α1 and α2 transcription in transformed nuclei was about 15 and 21%, respectively, of that obtained with control nuclei (Fig. 5, *Panel B*). These data suggest that reduced transcription of procollagen genes is a major factor in determining the levels of α1 and α2 messenger RNAs in RSV-transformed cells.

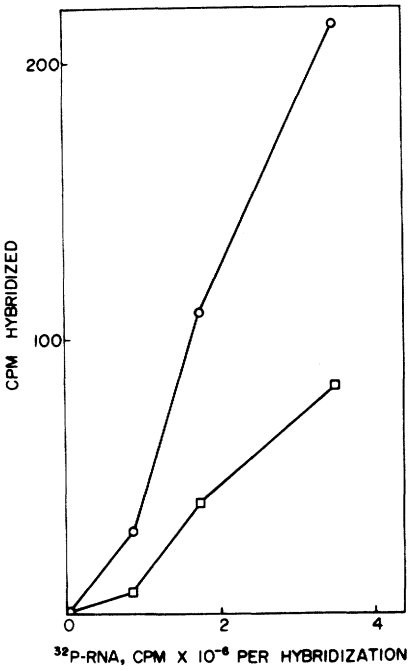

FIG. 4. **Hybridization of newly synthesized α1 and α2 procollagen RNA obtained by *in vitro* transcription of normal CEF nuclei.** Nuclei were isolated and pulsed *in vitro* in the presence of [^{32}P]UTP. RNA was isolated from nuclei and hybridized to pCg54(α1) (○——○) and pCg45(α2) (□——□) plasmids linked to filters as described under "Experimental Procedures."

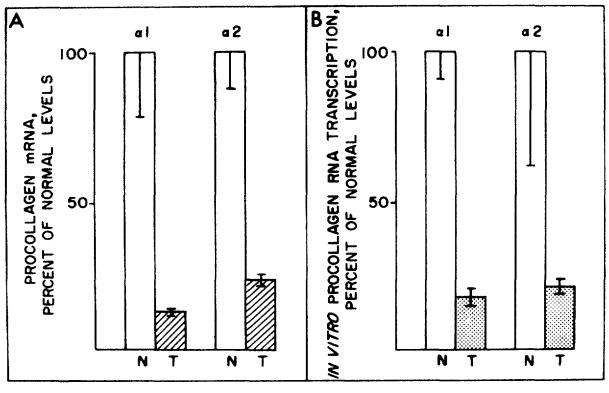

FIG. 5. **Comparison of α1 and α2 procollagen RNA synthesized *in vitro* with levels of cellular α1 and α2 mRNA in normal and RSV-transformed CEF.** Total RNA (*Panel A*) and nuclei (*Panel B*) were prepared from parallel cultures of normal (*N, open bars*) and transformed (*T, shaded bars*) CEF. α1 and α2 mRNA levels were determined as described under "Experimental Procedures." The data are normalized to 100% of normal cellular α1 and α2 procollagen mRNA levels or to 100% of the level of transcription by normal nuclei.

To explore the possibility that nuclear RNA degradation was a factor contributing to the reduction in the levels of newly synthesized procollagen RNA sequences in nuclei from transformed cells, we performed two types of experiments. Since a time course of total incorporation indicated that most rapid incorporation occurred during the first 10 min of transcription (Fig. 6*A*), we reasoned that procollagen mRNA-specific nuclease activity would be apparent as a decrease in parts per million hybridized to the procollagen plasmid filters with increasing time of transcription incubation. Nuclei from normal and transformed cells were incubated for 10, 20, and 30 min and their respective RNAs were isolated. No significant difference was observed in the parts per million of procollagen RNA hybridized over this time course (Fig. 6*B*).

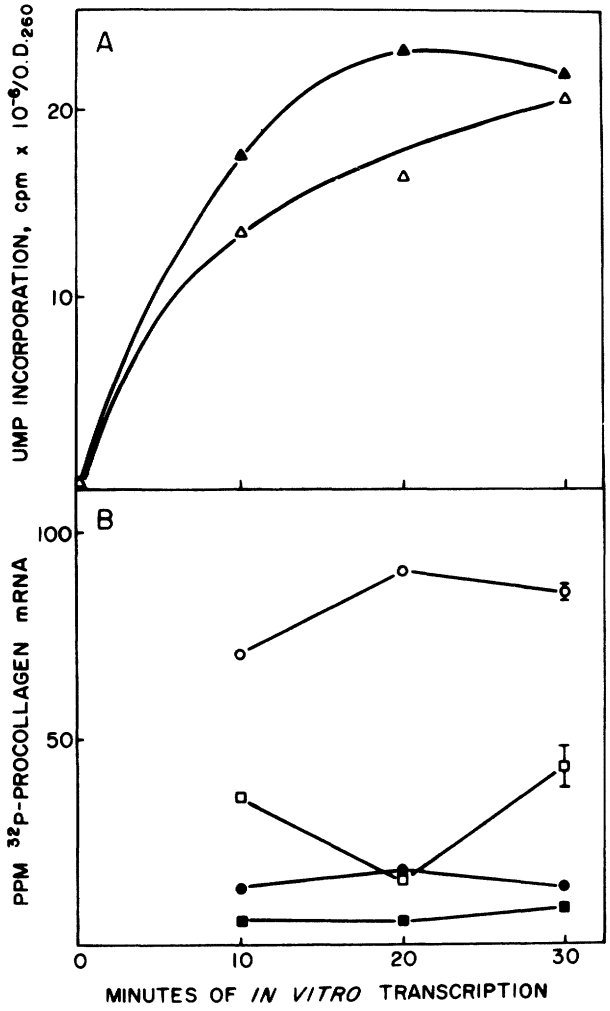

FIG. 6. **Total incorporation of UMP and relative levels of α1 and α2 procollagen mRNA over a time course of *in vitro* transcription.** Nuclei were prepared and transcribed as described under "Experimental Procedures." Total incorporation (*Panel A*) was determined for transformed (▲——▲) and normal (△——△) cell nuclei by spotting an aliquot of the transcription mixture onto Whatman 540 filter paper and precipitating in 5% trichloroacetic acid containing 10 mM PP_i. Relative synthesis of α1 RNA (◑——◑) and α2 RNA (◨——◨) in nuclei from normal (○, □) and transformed (●, ■) cells was determined by isolating newly transcribed RNA and hybridizing to pCg54(α1)- and pCg45(α2)-plasmid filters (*Panel B*). Normal nuclear RNA hybridizations contained 3×10^6 cpm and transformed nuclear RNA hybridizations contained 6×10^6 cpm each. Backgrounds were determined on blank filters and subtracted.

TABLE II

Effect of mixing normal and transformed nuclei on transcription of α1 and α2 RNA

Transcriptions performed for 30 min at 20 °C. Results are the average of two experiments.

Source of RNA	^{32}P hybridized to procollagen cDNA plasmids[a]			
	α1 plasmid	% control	α2 plasmid	% control
	cpm		*cpm*	
A. Normal and transformed nuclei; RNA isolated separately and mixed	112 ± 6	100	95 ± 8	100
Mixture of normal and transformed nuclei[b]	82 ± 8	73	107 ± 22	112
B. Normal and transformed nuclei, RNA isolated separately and mixed	122 ± 9	100	104 ± 4	100
Mixture of normal and transformed nuclei[c]	112 ± 23	91	122 ± 14	117

[a] Hybridization input was 4.8×10^6 cpm.
[b] Nuclei were mixed 10 min before transcription.
[c] Nuclei were mixed 10 min after transcription.

In the second experiment, described in Table II, nuclei from normal and transformed cells were mixed and incubated before or after the transcription labeling period. If nuclei from transformed cell or contaminating cytoplasm contained nucleases responsible for degrading procollagen transcripts, it seemed likely that we would observe a decrease in hybridization of RNA in preparations mixed both before (A) and after (B) the transcription reaction. If, on the other hand, the pp60^{src} protein somehow reduced transcription in the normal nuclei, this would be apparent in nuclei mixed before (A), but not after (B), the transcription labeling period. In order to determine the expected levels of hybridization, normal and transformed cell nuclei were allowed to transcribe separately; RNAs were isolated from each, and a mixture of normal and transformed cell nuclear RNA was hybridized to the plasmid filters. Some reduction in hybridization is apparent in the hybridization to the α1, but not to the α2, plasmid filters, by RNA from nuclei mixed before the transcription. No significant difference was observed when the nuclei were mixed after the transcription reaction. These experiments argue against the possibility that procollagen-specific mRNA degradation could account for the decrease in procollagen mRNA transcription observed in transformed nuclei.

DISCUSSION

Although it was previously known that *src* gene function is necessary for maintenance of the transformed state (for a review, see Ref. 14), this study shows that pp60^{src} activity can be correlated, in the course of a kinetic experiment, with the effect of viral transformation on the synthesis of a specific protein, collagen. Sefton *et al.* (47) reported that the extent of pp60^{src} activity, as measured by the γ chain phosphorylation assay, correlated generally with the state of transformation. They reported kinase activity in normal CEF at 2–3% and that in tsNY68-infected CEF at restrictive temperatures at 30–50% of the activity of fully transformed cells. In our studies, pp60^{src} kinase activity was also much higher in RSV transformants than in normal CEF. Our data with tsLA24-10-infected cells also showed significantly higher kinase activity, even at restrictive temperatures, than with uninfected cells and a 3- to 6-fold difference between pp60^{src} activity at restrictive and permissive temperatures (Fig. 1). This correlated with a 2- to 4-fold difference in the level of collagen synthesis between the tsLA24-10-infected cells at the restrictive and permissive temperatures. We observed no evidence for the requirement for a threshold level of pp60^{src} activity for onset of transformed characteristics.

These observations extend the emerging picture of the sequence of events which accompanies transformation. Although a time course of change in pp60^{src} activity has not been correlated with a specific transformation phenotype, Radke and Martin (49) have reported a phosphoprotein of 36,000 daltons which increases as soon as 20 min after shifting tsLA29-infected cells to permissive temperatures. At 4 h after the shift, this protein band was quite prominent, as was another band of 60,000 daltons, thought to represent the pp60^{src} protein. Changes in morphology have been reported within the first h after downshifting TaSp7- and tsNY68-infected cells (50). Actin cables were reduced within 3 h and surface fibronectin was greatly reduced by 4 h after temperature downshift. Hynes and Wyke (5) measured 2-deoxyglucose uptake in tsLA24A-infected cells and found that it was substantially increased by 2 h after shifting to permissive conditions. We found that the level of collagen synthesis changed within 4 h after the temperature shift to conditions permissive or restrictive for transformation. Pp60^{src} activity was also significantly different in temperature shift experiments by 4 h, when the first measurements were made. Activity continued to decrease or increase until between 24 and 36 h when it appeared to level off. This activity correlated inversely with the changes we observed in collagen synthesis.

The low levels of collagen synthesis in CEF after RSV transformation, about 14% of normal levels, closely parallel the decrease in procollagen mRNA levels. Hybridization assays revealed that transformed cell mRNA contained about 13% of the α1 sequences and 23% of the α2 sequences present in normal CEF (Fig. 5). The difference in mRNA levels between transformed and normal CEF is less than the 10-fold decrease in translatable procollagen mRNAs (9, 11) and the 20-fold decrease in procollagen mRNA sequences observed by Northern blotting with cloned cDNAs (10). That we observed a smaller decline in collagen specific mRNA sequences is probably due to the fact that our assay measures total (intact and partially degraded) mRNA sequences while the translation and blot assays measure unnicked, complete mRNA molecules.

We were interested in determining the mechanism of this decrease in procollagen mRNA levels. We began by examining the change in mRNA levels in tsLA24-10-infected cells shifted from a temperature restrictive to one permissive for transformation. Both α1 and α2 mRNA levels decreased most rapidly between 5 and 10 h after the temperature shift (Fig. 3). These results are in general agreement with the decrease in collagen synthesis and probably also reflect the increase in pp60^{src} activity. The change in procollagen RNA levels which accompanies transformation may be attributable to changes in procollagen mRNA synthesis since RNA isolated from transformed cell nuclei contained 15 and 21% of the level of newly synthesized α1 and α2 sequences, respectively, found in nuclei from normal cells. These reductions paralleled those in α1 and α2 mRNA in total RNA in transformed cells, suggesting that the decreased transcription of the type I procollagen genes is directly responsible for the observed reduction in α1 and α2 mRNAs.

Several experiments were performed to examine the possibility that pulse-labeled procollagen mRNA sequences were degraded in transformed nuclei. First, we demonstrated that the parts per million of labeled nuclear RNA which hybridized to α1 and α2 plasmids were constant over a 30-min period for preparations from both normal and transformed cells, despite the fact that synthesis is greatest early in the time course (Fig. 6). In a second control experiment, nuclei from normal and

transformed cells were mixed before or after transcription. When nuclear RNA, isolated separately from normal and transformed cells and from cells mixed before transcription, was compared, there was no significant difference in the extent to which the RNA hybridized to cDNA plasmids containing $\alpha 1$ or $\alpha 2$ sequences (Table II). We therefore concluded that procollagen-specific nucleases were not responsible for the decrease observed in procollagen RNA sequences synthesized in transformed nuclei. However, we currently cannot exclude additional regulatory elements which could function at the level of the processing of mRNA precursors or alter the stability of mRNA thereby affecting translational efficiency.

The RSV-transformed chicken cell offers a unique system for studying collagen regulation. We have observed an inverse correlation between collagen synthesis and $pp60^{src}$ kinase activity in these cells and have shown that transformation alters expression of the collagen genes at the level of transcription. We would like to know whether this change occurs at the level of chromosome structure and are attempting to determine whether the type I collagen genes have decreased DNase I sensitivity (51) after cellular transformation by RSV. We are also examining whether RNA polymerase II or high mobility group proteins 14 and 17, which confer DNase I sensitivity to actively transcribed genes (52, 53), are differentially phosphorylated upon transformation. Since there is a highly conserved homologous kinase in most higher eukaryotic cells, it would also be interesting to determine the correlation between this kinase and collagen synthesis at different cell densities in tissue culture or during development.

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