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Decrease in the levels of nuclear RNA precursors for alpha 2 collagen in Rous sarcoma virus transformed fibroblasts

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ABSTRACT

We have examined the levels of type I alpha 2 collagen RNA precursors, containing both intron and exon sequences in nuclear RNA preparations of chick embryo fibroblasts (CEF) and of CEF transformed by the Schmidt-Rupin strain of Rous sarcoma virus (RSV). We have used two different fragments of chick alpha 2 collagen genomic DNA as hybridization probes in S1 mapping experiments. Each of these DNA probes contains an entire intron. Our results indicate that the levels of the primary transcript of alpha 2 collagen RNA are much lower in RSV-CEF than in CEF. They suggest, but do not prove that the effect of the transforming protein $p60^{src}$ on the synthesis of alpha 2 collagen is mediated by a transcriptional control mechanism.

INTRODUCTION

Transformation of chick embryo fibrocblasts (CEF) by Rous sarcoma virus (RSV) causes a number of cellular changes. These changes can be subdivided into at least three groups (a) alterations in cellular growth control, which are reflected by the ability of such cells to produce tumors in animals; (b) alterations in the adhesive properties of the cells manifested by changes in cell shape, motility, cytoskeleton, number of surface ruffles and microvilli, etc. (for review see ref. 1); (c) changes in the rate of synthesis of a discrete group of proteins. Two proteins whose synthesis is markedly decreased are fibronectin (2) and type I collagen (3). These changes are specific since the synthesis rate for most other cellular proteins is unaltered.

Type I collagen is the major collagen which is synthesized by CEF. It is composed of two alpha 1 subunits and one alpha 2 subunit. Using cloned alpha 1 and alpha 2 collagen cDNAs and cloned fibronectin cDNA as hybridization probes (4,5,6), we recently demonstrated that the decreased synthesis rate for type I collagen and for fibronectin in RSV transformed

CEF was due to a reduction in the copy number of alpha 1 and alpha 2 collagen mRNA and of fibronectin mRNA (7,8,6). Similar results were reported by others using uncloned collagen cDNA preparations (9,10). By performing experiments with a mutant of RSV, temperature sensitive for transformation, we showed that the product of the src gene of RSV, p60^{src} was responsible for the coordinate reduction of these three mRNAs (8,6).

Possible causes for the reduction in the levels of alpha 1 and alpha 2 collagen mRNAs and fibronectin mRNA in RSV transformed cells include (a) transcriptional shut-off via repressor-like or other mechanisms caused or induced by RSV transformation; (b) alterations in one or several steps in the processing of the primary transcripts; (c) increased degradation rate for the translatable fully processed forms of these mRNAs. In each case the mechanism needs to be very specific. We show here that the levels of the primary transcript of the alpha 2 collagen gene containing both intron and exon sequences are reduced in RSV transformed cells. This result strongly suggests that the effect of p60^{src} on type I collagen synthesis is mediated by a transcriptional control mechanism.

MATERIALS AND METHODS

Preparation of DNA probes

A 1.7 Kb Hind III fragment, which is derived from lambda COL-204, a genomic clone containing the 3' end of the chick type I alpha 2 collagen gene (12), was cleaved with endonuclease Ava II and the products fractionated by polyacrylamide gel electrophoresis. Two fragments, one of 156 bp, the other of 256 bp, were isolated from the gel (16). The 156 bp fragment was labeled at its 5' ends, using T4 polynucleotide kinase, and its strands were separated (16). The (+) and (-) DNA strands were hybridized to RNA extracted from the nuclei of normal CEF and CEF transformed with the Schmidt-Ruppin (SR) strain of RSV. The 256 bp Ava II fragment was labeled at its 3' ends with terminal transferase (12) and its strands were separated. The (-) DNA strand was hybridized to nuclear RNA and SR-RSV transformed CEF.

Preparation of nuclear RNA

The nuclear RNA was prepared from sucrose gradient purified nuclei, according to a procedure described by Lerner et al. (14). These preparations contained a considerable portion of very large RNAs as assayed by electrophoresis on methylmercury agarose gel.

S1 nuclease mapping

10-20 ng of separated DNA strands, labeled either at their 5'

(2 x 10^6 cpm/pmole end) or at their 3' end (5 x 10^5 cpm/pmole end) were incubated with the amounts of nuclear RNA indicated in the figure legends in 80% formamide, 0.03 M Pipes (pH 6.5), 1 mM EDTA, 0.4 M NaCl, at 75°C for 5 minutes and then at 50° overnight. The hybridization reactions in a final volume of 0.03 ml, covered with a drop of paraffin oil, were performed in a 1.5 ml Eppendorf tube. At the end of the hybridization period, reactions were treated with 1,000 U/ml of S1 nuclease (a gift of Dr. J. Alwine, NIH) (17). The samples were extracted with phenol-chloroform-isoamyl alcohol (25-24-1), precipitated with ethanol, dissolved in 80% formamide, heated at 90°C for 2 minutes and electrophoresed in a 10% polyacrylamide gel containing 7 M urea. Autoradiography with an intensifier screen was at -70° for 1-2 days.

Cells

CEF were prepared from 10-day-old embryos and infected with SR-RSV. The cells were passed twice after infection, expanded to 150 mm dishes and harvested two days after the last transfer. All infected cultures showed the disordered growth pattern and cellular rounding characteristic of SR-RSV infected CEF.

RESULTS

We have recently isolated a series of overlapping recombinant genomic clones which cover the chick alpha 2 collagen gene. The gene is at least 38 Kb in length. Its coding information is subdivided into more than 50 exons which are interrupted by introns of various sizes (11). The structure of the gene implies that the conversion of the primary transcript of the alpha 2 collagen gene to fully processed translatable cytoplasmic messenger RNA, includes at least 50 splicing events (11). The size of the mature translatable form of alpha 2 collagen mRNA is 5,000 nucleotides. Given the very large number of intervening sequences, it is obvious that it would be very difficult to detect discrete RNA precursors by hybridization-blotting methods. We therefore decided to measure the levels of nuclear alpha 2 collagen RNA precursors by S1 mapping using as probe genomic DNA segments containing intron sequences. By this method we have been able (a) to detect unspliced precursor RNA for alpha 2 collagen and (b) to demonstrate the stepwise removal of some introns in the alpha 2 collagen gene (12).

Lambda COL-204 is a clone which contains the 3' portion of the alpha 2 collagen gene (13). Figure 1 shows a restriction map of a 1.7 Kb Hind III

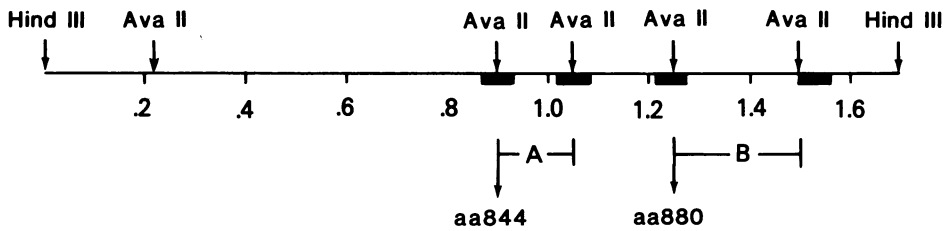

Figure 1. Alpha 2 collagen specific genomic DNA fragments used as hybridization probes. The 1.7 Kb Hind III fragment is derived from lambda COL-204, a genomic clone containing the 3' end of the chick type I alpha 2 collagen gene (12). The boxes represent the exons present in this fragment. The amino acid residues present at the beginning of the two fragments used as hybridization probes are indicated (V.E.A. unpublished data; P. Fietzek, personal communication). (A) represents a 156 bp fragment with Ava II termini; it contains, beginning at the 5' end, a 35 bp exon sequence encoding a.a. residues 844-856 of the alpha 2 collagen protein, a 90 bp intron and a 31 bp exon sequence encoding a.a. residues 857 to 866. (B) is a 256 bp Ava II fragment which contains, beginning at the 5' end, a 35 bp exon sequence coding for a.a. residues 880 to 891, a 215 bp intron and 4 bp exon sequence corresponding to a.a. residues 892-893.

fragment, located at the 5' end of this clone. The fragment contains 4 exons. Two Ava II subfragments of this 1.7 Kb Hind III fragment, one of 156 bp, the other of 256 bp, were used as hybridization probes. The 156 bp Ava II fragments (A in Fig. 1) contains, beginning at the 5' end, a 35 bp exon sequence coding for amino acid (a.a.) residues 844 to 856 of the alpha 2 collagen protein, a 93 bp intron and a 31 bp exon sequence coding for residues 856 to 866 (V.E.A., unpublished results, P. Fietzek, personal communication). The 256 bp Ava II fragment (B in Fig. 1) contains, beginning at the 5' end, a 35 bp exon sequence coding for residues 880 to 891, a 215 bp intron and a 4 bp exon sequence corresponding to residues 891 and 892 (V.E.A., unpublished results; P. Fietzek, personal communication).

Figure 2 shows the results of an experiment in which the (-) strand of the 156 bp Ava II fragment, labeled at its 5' end, was hybridized to nuclear RNA from CEF and from RSV transformed CEF. After the hybridization period, the hybrids are treated by S1 nuclease and the labeled probe is examined by gel electrophoresis (12). Both nuclear RNA preparations from normal CEF and from RSV transformed CEF exhibit a similar RNA size distribution when examined by electrophoresis on denaturing agarose gels (data not

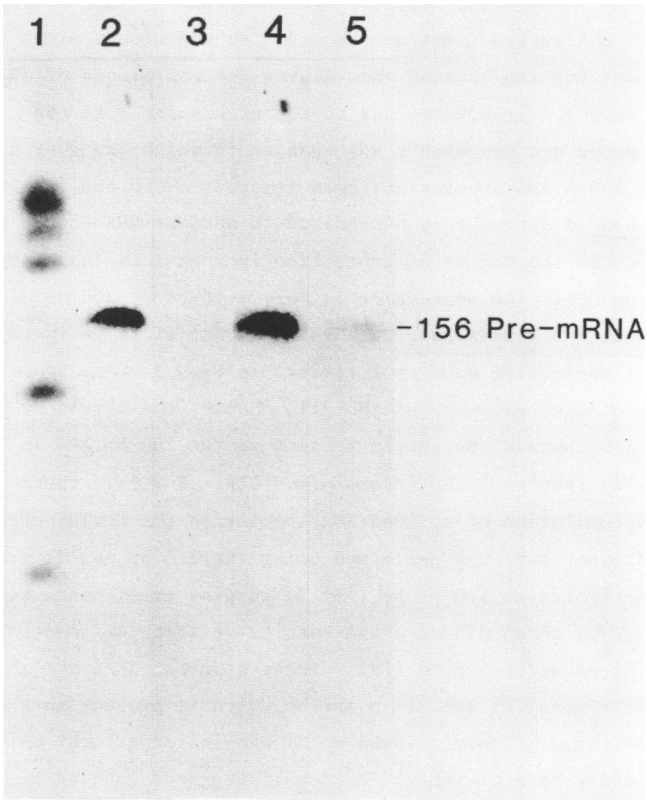

Figure 2. Identification of alpha 2 collagen RNA species containing the 156 n Ava II sequences in nuclear RNA preparations from normal and RSV transformed chick embryo fibroblasts. Lane 1, size markers, 5' end labeled ΦX174 DNA fragments (cleaved with endonuclease Hae III); lane 2, (-) DNA strand of the 156 n Ava II fragment hybridized with 100 ug/ml of yeast RNA, no S_1 nuclease treatment; lane 3, 156 n Ava II (-) DNA strand hybridized with 300 ug/ml of yeast RNA, treated with S_1 nuclease; lane 4, 156 n Ava II (-) DNA strand hybridized with 30 ug/ml of nuclear RNA from normal CEF treated with S_1 nuclease; lane 5, 156 n Ava II (-) DNA strand hybridized to 30 ug/ml of nuclear RNA from the Schmidt-Ruppin (SR) strain of transformed CEF and treated with S_1 nuclease.

shown). Hence, the overall degradation rate of total nuclear RNA is not substantially different in these two cell types. Nuclear RNA (30 ug/ml) from normal CEF clearly protects the (-) DNA strand of the 156 bp Ava II fragment from S1 digestion. This protection is dependent on the presence

of RNA (see lanes 3 and 4); it is also strand specific since no protection occurs with (+) DNA strand (data not shown). An additional minor species of 31 nucleotides (n) can be seen when higher concentrations of RNA are used (not shown). It corresponds to the exon located at the 5' end of the probe and represents RNA species in which the intron present in the 156 n RNA species has been removed. When the (-) DNA strand of the 156 bp Ava II fragment is hybridized to nuclear RNA (30 ug/ml) from RSV transformed CEF the degree of protection is much less than with nuclear RNA from CEF. The protection is more evident at 500 ug/ml but does not approach the levels seen with RNA from normal cells (data not shown). The 31 nucleotide band (not visible in Fig. 2, Lane 5) is detectable only after a prolonged exposure of the gel. Hence, the levels of nuclear RNA species which contain the intron present in the 156 bp Ava II fragment are substantially reduced in RSV transformed CEF. Further, there is no evidence for accumulation of spliced RNA species in the nuclei of RSV-CEF.

A similar experiment was performed using the 256 bp Ava II fragment (B of Figure 1) as hybridization probe. By S1 mapping experiments we have previously observed three discrete RNA species in which defined portions of the 216 n intron were deleted (12). These discrete RNAs are intermediates in the stepwise removal of the 216 n intron which is present in the 256 bp Ava II fragment (12). Figure 3 shows an S1 mapping experiment using as probe the (-) DNA strand of the 256 bp Ava II fragment labeled at its 3' end. With nuclear RNA from normal CEF we detect a 256 n species which represents the unspliced precursor RNA but also bands of 210 n, 196 n, and 186 n representing RNA species in which defined parts of the 216 n intron have been eliminated (12). After a long exposure of the autoradiograph, a 35 n band also appears. This 35 n band corresponds to an RNA species in which the entire 216 n intron has been removed. The 256 n Ava II probe was labeled at its 3' end because the intermediate precursors are only observed when this probe is labeled at the 3' end and not when the probe is labeled at its 5' end. With nuclear RNA from RSV transformed CEF, the same hybridization pattern is observed but the relative levels of each protected species are very much reduced and difficult to reproduce photographically (lane 5).

With both the 156 n and 256 n probes, the ratio of precursor RNA to mRNA is similar in nuclear RNA preparations of normal and RSV transformed CEF. Our results clearly indicate a pronounced decrease in the levels of the intron present in the 256 bp fragment in RSV transformed

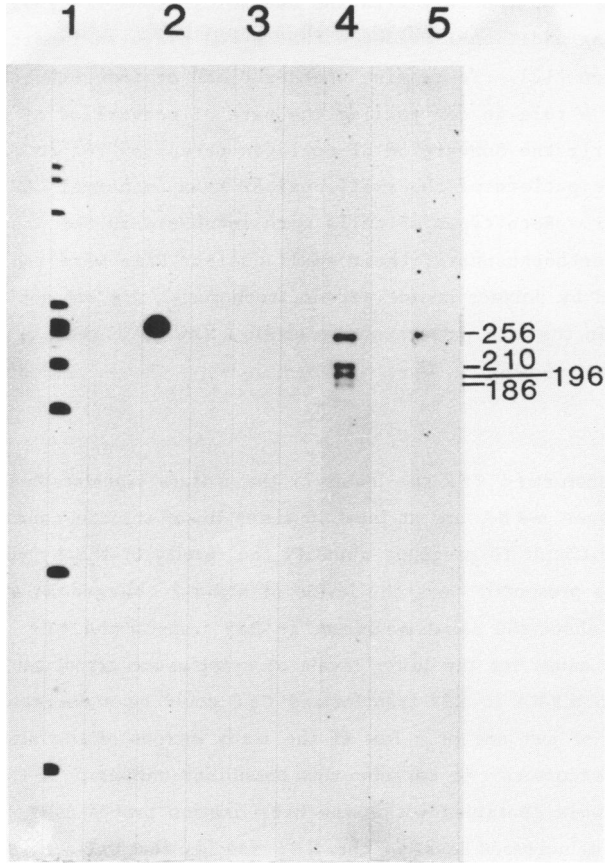

Figure 3. Identification of alpha 2 collagen RNA precursors containing the 256 n Ava II sequences in nuclear RNA preparation from normal and RSV transformed CEF. Lane 1, DNA size markers, 5' end labeled ΦX174 DNA fragment (cleaved with endonuclease Hae III); lane 2, 256 n Ava II (-) DNA strand hybridized to 300 µg/ml of yeast RNA, no S_1 treatment; lane 3, 256 n Ava II (-) DNA strand hybridized to yeast RNA (300 µg/ml) and treated with S_1 nuclease; lane 4, 256 n Ava II (-) DNA strand hybridized to nuclear RNA from normal CEF (150 µg/ml) and treated with S_1 nuclease; lane 5, 256 n Ava II (-) DNA strand hybridized to 300 µg/ml of nuclear RNA from SR-RSV transformed CEF and treated with S_1 nuclease.

CEF, without detectable accumulation of intermediary precursors.

It has recently been proposed that small nuclear RNAs, like U_1RNA, were involved in the RNA splicing reaction, because the 5' end of U_1RNA

was found to be complementary with the ends of introns (14,15). We have presented strong additional evidence that U_1RNA plays an important role in this reaction (12). To examine whether U_1RNA or some other small nuclear RNA might play a role in controlling the rate of conversion of precursor RNA, particularly the conversion of collagen precursor RNA to mature mRNA, we compared the pattern of the small nuclear RNAs in normal CEF and RSV transformed CEF. Both types of cells were incubated in the presence of ^{32}[P]-labeled orthophosphate, their small nuclear RNAs were isolated (14) and fractionated by polyacrylamide gel electrophoresis. We did not detect any difference in the size pattern of the small RNAs or in their relative amounts (data not shown).

DISCUSSION

In RSV transformed CEF the levels of the mature translatable species of alpha 2 collagen mRNA are at least 10 times lower than in normal CEF (7). Although it is difficult to precisely quantify the results of the hybridization experiments presented here, the levels of alpha 2 collagen RNA precursors are reduced by about the same magnitude in RSV transformed CEF.

A possible cause for the lower levels of cytoplasmic translatable alpha 2 collagen mRNA in RSV transformed CEF could be a decrease in the rate of splicing of just one or a few of the many introns of the alpha 2 collagen RNA precursor. We consider this possibility unlikely. If these more slowly spliced introns were contained within the hybridization probes used, we would expect to see an increased level of the RNA species containing these introns. On the other hand, if the introns, postulated to be removed more slowly, were not contained within the hybridization probes, we would anticipate (a) that the levels of alpha 2 collagen RNA species containing the other introns would be the same in normal and RSV transformed CEF, and (b) that alpha 2 collagen RNA species, in which most or all of the other introns had been removed, might accumulate in the nucleus. None of these predictions were observed in our experiments.

Other possible explanations for the reduced levels of precursor RNA for alpha 2 collagen in RSV transformed CEF include: an increase in the rate of both processing and degradation for this specific RNA species in these cells; or, an increase in the rate of transport of precursor RNA for alpha 2 collagen from nucleus to cytoplasm followed by rapid degradation of this RNA. We have no data to evaluate these possibilities.

Our experiments indicate that in RSV transformed CEF the levels of

alpha 2 collagen RNA precursors are substantially reduced. We find no accumulation in the nucleus of spliced RNA species in which introns have been partially or entirely removed. We believe these results suggest that there is a reduction in the rate of transcription of the alpha 2 collagen gene in RSV transformed CEF.

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