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Journal
Cancer Research, 41(3)

ISSN
0008-5472

Authors
Sandmeyer, S
Smith, R
Kiehn, D
et al.

Publication Date
1981-03-01

[CANCER RESEARCH 41, 830–838, March 1981]
0008-5472/81/0041-0000$02.00

Correlation of Collagen Synthesis and Procollagen Messenger RNA Levels with Transformation in Rat Embryo Fibroblasts[1]

Suzanne Sandmeyer,[2] Ronald Smith, Donald Kiehn, and Paul Bornstein[3]

Department of Biochemistry [S. S., P. B.] and Microbiology [R. S., D. K.], University of Washington, Seattle, Washington 98195

ABSTRACT

A line of normal rat embryo fibroblasts was transformed with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (a chemical carcinogen), SV40 and polyoma virus (two DNA viruses), and Rous sarcoma virus (an RNA tumor virus). In this study, we report a comparison of the levels of collagen synthesis and procollagen messenger RNA (mRNA) in 13 lines selected after transformation with one of these agents. Collagen synthesis and procollagen mRNA levels were compared with the degree of transformation determined from morphology, saturation density, growth in agarose, and tumorigenicity in nude mice. Each class of transformants had a characteristic level of collagen synthesis; this level correlated inversely with the degree of transformation of the rat embryo fibroblasts. In *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and SV40 transformants which were moderately transformed, collagen synthesis was hardly affected, but, in polyoma virus and Rous sarcoma virus transformants which were more severely transformed, collagen synthesis was 30 to 48% and 12 to 25%, respectively, of control levels. Type I procollagen mRNA activity measured in RNA from nine of the lines by an *in vitro* translation assay also decreased with increasing severity of transformation. Procollagen mRNA levels were reduced to about one-half of control levels in one SV40 transformant and to 17 to 23% of controls in polyoma virus and Rous sarcoma virus transformants. We conclude that, in this series of rat fibroblast lines, transformation with different agents resulted in characteristic levels of collagen synthesis and that collagen synthesis was most reduced in the cells which were most transformed by other criteria.

INTRODUCTION

Fibroblasts undergo a variety of complex changes during transformation. At the cellular level, they become rounded and disorganized (34), grow to a higher saturation density (24), show less dependence on serum (for review, see Ref. 35), and acquire the ability to grow in agarose (27). Biochemically, decreased cAMP[4] levels (32), increased transport of glucose (16), increased hyaluronate synthesis (19), decreased surface fibronectin (for a review, see Ref. 17), changes in glycoproteins and glycolipids (14, 18), and decreased collagen synthesis (13, 15, 20, 23, 28, 29, 31) have been reported. Transformed cells may also have tumorigenic potential in animals of the same or related species.

The pleiotropic nature of transformation which results in numerous differences between normal and transformed cells also acts to obscure the mechanism of particular changes. For instance, it has long been known that a decrease in collagen synthesis may accompany fibroblastic transformation, yet the relationship of collagen biosynthesis to cellular transformation is not clear. In addition to a decrease in collagen synthesis, changes in collagen types (15) and possibly levels of procollagen peptidase (2) and differences in prolyl hydroxylase activity (23) and ascorbate dependence of collagen synthesis (11, 13) have been observed. If the mechanism by which transformation affects these processes could be determined, the coordination of different steps in collagen biosynthesis could be better understood. In order to explore the relationship of collagen biosynthesis to transformation, we asked 3 specific questions. (*a*) Is the effect of transformation on collagen biosynthesis a function of the transforming agent? (*b*) Is this effect proportional to the degree of transformation, as determined by other parameters? (*c*) How are changes in the level of collagen synthesis mediated during transformation caused by different transforming agents?

We compared collagen synthesis and procollagen mRNA levels in 2 normal rat embryo fibroblast lines and 13 transformed lines which had been derived previously from the normal lines by treatment with one of 4 transforming agents, MNNG, SV40, PY, or RSV. These cell lines were evaluated in previous work[5] for transformation on the basis of growth in agarose, saturation density, and tumorigenicity. In this study, we found that different transforming agents produced lines with different levels of collagen synthesis. The PY and RSV cell lines, the 2 most transformed cell types by the criteria of growth in agarose, saturation density, and tumorigenicity, were also most reduced in collagen synthesis. In all but one of 11 cases examined, a reduction in the level of collagen synthesis was accompanied by a significant reduction in the level of translatable procollagen mRNA.

MATERIALS AND METHODS

Cells and Culture Conditions. The experiments described here were performed with an early-passage rat fibroblast strain, a diploid rat embryo fibroblast line, 3Y1 B Cl 1-6 (3Y1), cloned from low-passage rat embryo cultures by Kimura *et al.* (21), a normal subclone of this line (N2), and viral and chemically transformed clonal derivatives of these lines. These lines and clones will be described elsewhere[5]; their properties are summarized in Table 1. Normal cells were characterized by density-dependent growth, high serum requirement, inability to form dense colonies on plastic, no oncogenic potential as assayed

[1] This work was supported by NIH Grants AM 11248 and DE 02600.

[2] Present address: Department of Genetics, Washington University, 660 S. Euclid, St. Louis, Mo. 63110.

[3] To whom requests for reprints should be addressed.

[4] The abbreviations used are: cAMP, cyclic adenosine 3′:5′-monophosphate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PY, polyoma virus; RSV, Rous sarcoma virus; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate.

Received June 30, 1980; accepted November 24, 1980.

[5] D. Kiehn, S. Martin, and R. Smith. Dependency of the phenotype of transformation: specificity of Rous sarcoma virus, submitted for publication.

Table 1

Characteristics of normal and transformed rat embryo fibroblasts

Transforming agent	Cell type	Saturation density[a]	Growth in agarose[b]	Tumor incidence[c]	Mean latent period of tumor development[d]
None	3Y1	0.54	0.001	0/4	
	N2	0.45	0.001	0/5	
MNNG	NG-8	0.61	1.08	0/3	
	NG-E	0.48	0.14	0/2	
SV40	SV-1	2.7	2.7	5/6	46 (42–50)
	SV-3	0.73	0.97	3/3	61 (51–70
PY	PY-2P2	4.5	6.7	3/3	7
	PY-2N	5.9	5.7	3/3	16
	PY-3	4.7	6.8	3/4	29 (21–40)
RSV	RS-4N	1.0	3.1	2/2	10
	RS-4	3.6	+++	2/2	7 (6–8)
	RS-1	3.5	+++	4/4	6
	RS-6	3.1	4.2	2/2	13
	RS-5	2.5	8.0	2/2	6

[a] Cultures were seeded at 10^5 cells/35-mm plastic dish in 2 ml DMEM containing 1.5% fetal calf serum. The medium was changed, and the cells were counted daily until a constant cell number was achieved. Saturation density is defined as the maximum number of cells × 10^6 per 35-mm dish.

[b] Numbers indicate the percentage of cells forming colonies in soft agarose. Each 5-cm plate contained 5 ml of 0.55% agarose in DMEM plus 10% fetal calf serum and was overlaid with 1.5 ml of 0.36% agarose in this medium containing 10^5 cells. On Days 1, 8, and 15, the spent liquid medium was discarded, and 5 ml of DMEM plus serum were added. On Day 21, colonies larger than 103 μm in diameter were scored microscopically with a calibrated grid reticle. +++, too many colonies to count.

[c] Ratio of mice with tumors to total inoculated. Mice 2 to 4 months of age were inoculated s.c. in the scapular region with 2 × 10^6 cells/0.2 ml phosphate-buffered saline and maintained in germ-free isolation. All negative animals were observed for 8 months.

[d] The latent period is the time in days between inoculation and the first appearance of a palpable tumor mass. The range is in parentheses. For values without reported range, all animals first demonstrated the tumor on the same day.

in adult nude mice, and anchorage dependence. After treatment of normal cells with a chemical carcinogen, MNNG, or with a transforming virus, SV40, PY, or RSV, transformants were isolated as discrete colonies appearing in agarose.[5] Chart 1 describes the clonal derivation of particular lines used in these studies. In addition to being able to grow in agarose, transformants grew to high saturation densities, had low serum dependence, formed dense colonies on plastic, and had tumorigenic potential in nude mice. Although the absolute values for saturation density and growth in agarose reported in Table 1 differed from experiment to experiment, the relative values remained much the same.

Cells were maintained in DMEM containing 10% fetal calf serum (North American Biologicals, Inc.) supplemented with 60 μg penicillin per ml, 100 μg streptomycin per ml, and 50 units mycostatin per ml at 37° in a 10% CO_2 atmosphere. Cell numbers were determined by Coulter counter determinations on cells suspended by trypsinization at the time of the experiment. Cell doubling times were all between 18 and 24 hr. Experiments were performed on cells grown for less than 6 weeks from frozen stocks of characterized lines, during which time no changes were observed in cellular morphology or behavior. All experiments were carried out on subconfluent cells in order to avoid variations due to the growth phase of the cells being examined.

Protein Determination. The protein content of cells was determined by the assay of Lowry *et al.* (25).

SDS-Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis was as described by Laemmli (22). For protein analysis, gels were stained with 0.25% Coomassie Brilliant Blue R (Sigma Chemical Co.) in 20% trichloroacetic acid, 45% methanol, and 9% acetic acid. Radiolabeled protein gels were prepared for fluorography according to Bonner and Lasky (6). Stained gels and fluorograms were scanned in their linear range on a Quick Scan II densitometer (Helena Laboratories).

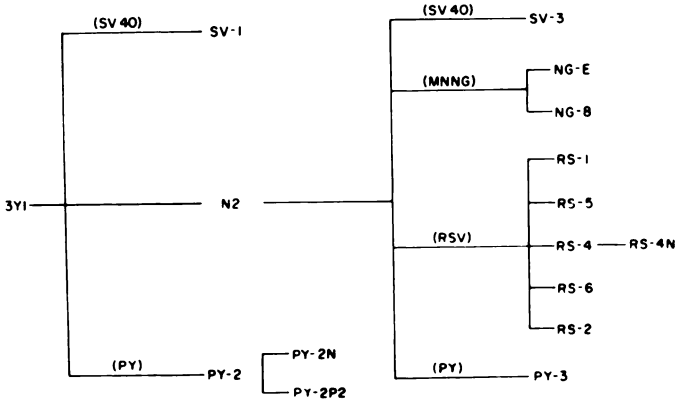

Chart 1. Derivation of transformed rat embryo fibroblast clones. Normal rat embryo clones, 3Y1 and N2, were transformed with MNNG, SV40, PY, and RSV. Transformed clones were selected by culturing in agarose.

Collagen Synthesis. Collagen synthesis in pulse-labeled cell layer and medium proteins was determined by collagenase assay (26). Cells were plated at a density of 5 × 10^4 cells/35-mm plate, and the medium was changed on the following day. On the third day, cells in 2 duplicate plates were preincubated in DMEM supplemented with 50 μg ascorbate per ml, penicillin, and streptomycin as described above for 1 hr. Cells were then labeled for 30 min or 4 hr with 25 μCi of L-[2,3-^{3}H]proline or L-[2,3-^{3}H]alanine per ml. After the 4-hr labeling period, the medium was decanted, spun to remove cellular debris, and frozen at −20° until it was processed.Cell layers were washed 3 times with phosphate-buffered saline and frozen at −20° until they were processed. Cell layers were solubilized in 0.5 M ammonium hydroxide and precipitated by making the solution 10% in trichloroacetic acid. All other procedures and the assay using collagenase digestion were as described by Peterkofsky and Diegelmann (26). The collagenase used in these studies (Form III; Advanced Biofactures) was shown to be free of nonspecific proteolytic activity by its inability to digest tryptophan-labeled cell layer proteins (less than 0.5% digestion) under the conditions of the assay.

Translation Assay for Procollagen mRNA. Total RNA from one or more representatives of each class of normal and transformed cells was extracted from 8 subconfluent 135-mm tissue culture dishes as described by Rowe *et al.* (29). Procollagen mRNA activity was assayed by translation of this RNA in a reticulocyte lysate system optimized for type I procollagen synthesis and by immunoprecipitation of the translated type I procollagen (29). Type I protocollagen standards were obtained by labeling N2 cells as described in the presence of α,α′-dipyridyl. Subconfluent cultures were labeled with 25 μCi of L[2,3-^{3}H]proline per ml for 6 hr in DMEM supplemented with 15.5 μg α,α′-dipyridyl per ml (9), penicillin, and streptomycin as above.

Determination of Specific Activity of Normal and Transformed Cell Protein Labeled with [^{3}H]Proline. Sixteen 135-

mm tissue culture plates of subconfluent PY-3 and RS-1 cells and 10 plates of N2 cells were labeled with [2,3-^{3}H]proline as described above for 8 hr. Medium was removed at the end of the period and centrifuged to remove debris; part of each sample was precipitated in 10% trichloroacetic acid, washed with ethanol and ethanol-ether, and processed for SDS-polyacrylamide gel electrophoresis. The remaining medium samples were precipitated and washed as described above. Samples were then processed for hydroxyproline analysis as described by Crouch and Bornstein (8). Samples were assayed in parallel for ninhydrin-staining material and radioactivity.

Pepsin Digestion. Preparation and pepsin digestion of medium and cell layer samples were as described (30). Pepsin digestion was stopped with the addition of a 10-fold M excess of pepstatin A. Digests were clarified by spinning at 10,000 rpm for 10 min in Sorvall HB-4 rotor. The solution containing the pepsin-resistant material was brought to 10% NaCl and stirred for several hr. Following an overnight precipitation, samples were again centrifuged for 20 min at 10,000 rpm. Pellets were washed with cold 95% ethanol and lyophilized. Proteins were then dissolved in sample buffer for electrophoresis.

Immunoprecipitation. Immunoreactions were carried out with rabbit anti-rat type I procollagen IgG which had been purified by ammonium sulfate precipitation and DEAE chromatography as described by Campbell *et al.* (7) and with a preimmune globulin fraction purified by ammonium sulfate precipitation. Rabbit antibodies were precipitated with an excess of sheep anti-rabbit serum or with an excess of fixed *Staphylococcus aureus* (12).

Supplementation of Culture Medium with Proline. N2, PY-3, and RS-1 cells were plated and fed on 2 successive days with DMEM supplemented with 10% fetal calf serum, penicillin, and streptomycin as described previously and with 0, 0.1, 0.5, 1.0, 5 or 10 mM L-proline. On the fourth day, the cultures were preincubated with fresh medium supplemented with 50 μg ascorbate per ml. Cells were labeled with 25 μCi of [^{3}H]alanine per ml for 30 min. Cell layers were washed and processed for collagenase assay as described previously.

RESULTS

Cell Transformation. The transformation and cell culture characteristics of the cells used in this study have been described elsewhere.[5] One early-passage rat fibroblast strain was studied. The other cell lines were ultimately derived from the normal progenitor clone, 3Y1 (21). The 3Y1 or N2 lines were transformed with 4 different transforming agents: MNNG; SV40; PY; or RSV. Chart 1 shows the derivation of each clone. We examined collagen synthesis in 2 normal lines, 3Y1 and N2; 2 MNNG transformants, NG-8 and NG-E; 2 SV40 transformants, SV-1 and SV-3; 4 PY transformants, PY-2P2, PY-2N, PY-2, and PY-3; 5 RSV transformants, RS-4N, RS-4, RS-1, RS-6, and RS-5; and an RSV transformation revertant, RS-2.

Fig. 1 shows the morphology of representatives of each of these classes of cells in monolayer culture. In *A* and *B* are normal cultures, LRE and N2, respectively. Note the alignment of the N2 cells. The *center* shows 2 examples of moderately transformed lines. *C* is a MNNG transformant, NG-8; these cells had lower saturation densities than did other transformants, did not grow as well in agarose, and did not cause tumors when injected into nude mice. However, this MNNG transformant is more rounded and disorganized than the normal cells. *D* shows SV-3, one of the SV40 transformants; these cells have a somewhat higher saturation density in low serum than do the MNNG transformants and cause tumors when injected into nude mice. Morphologically, the SV-3 cells are somewhat rounded but not strikingly disorganized.

The *bottom* shows 2 examples of the most transformed cells examined in this study. The PY clone, PY-3, shown in *E* is very transformed as indicated by saturation density, growth in agarose, and tumorigenicity. Morphologically, these cells typify the rounded almost cobblestone appearance of all PY transformants in monolayer culture. An RSV transformant, RS-1, is shown in *F*. Although these cells do not have as high a saturation density as PY transformants, they do cause tumors which grow more rapidly in nude mice than the corresponding tumors caused by PY transformants. The RSV tumors are very unusual and are discussed below in relation to the biochemical properties of the cells. Morphologically, the RSV transformants are unique, presenting a very rounded appearance and showing clustering during growth.

Proteins Synthesized by Normal and Transformed Rat Embryo Fibroblasts. Representative lines of normal cells and MNNG, SV40, PY, and RSV transformants were pulse labeled with [^{3}H]proline, and cell and culture medium proteins were electrophoresed as described in "Materials and Methods." The labeled proteins are shown in Fig. 2, *A* and *B*, respectively. The cell layer pattern, although complex, reveals 2 major bands migrating in the positions of the proα1(I) and proα2 chains of type I procollagen. These 2 proteins also appeared as major bands in the medium pattern of most of the cell lines examined. These bands were collagenase sensitive as were 2 fainter higher-molecular-weight bands, which were most distinct in the culture medium of the PY transformants. The RSV transformants have much less secreted protein than do any of the other cell types, and almost no type I procollagen was observed. The one revertant examined, RS-2, appears to have unusually high levels of type I procollagen (Fig. 2, *A* and *B*, *Lane 9*).

Collagen Synthesis and Secretion by Normal and Transformed Rat Embryo Fibroblasts. Representatives of each normal line and each group of transformants were pulse labeled for 4 hr as described in "Materials and Methods," and medium and cell layer proteins were analyzed by collagenase assay (26). The percentage of protein synthesis which was collagenous in each case is shown in Table 2. The collagenase results quantitate and extend the observations made by polyacrylamide gel analysis. The normal cells, low-passage rat embryo fibroblasts, 3Y1, and N2 synthesized different levels of collagen, but in each case more than 1.6% of total protein was collagenous. The N2 cells which had been in culture the longest synthesized the least amount of collagen of the normal cells tested. The one revertant clone examined, RS-2, synthesized a higher level of collagen than either of the normal lines. Each of the SV40-transformed lines synthesized levels of collagen that were close to the level of its normal progenitor.

Lines cloned after transformation with SV40, MNNG, PY, and RSV fell into 3 classes of collagen synthesis. MNNG- and SV40-transformed clones made a normal or slightly reduced level of collagen. The 4 PY transformants, which were more transformed by the criteria of saturation density, growth in agarose, and tumorigenicity, synthesized between 30 and 48%

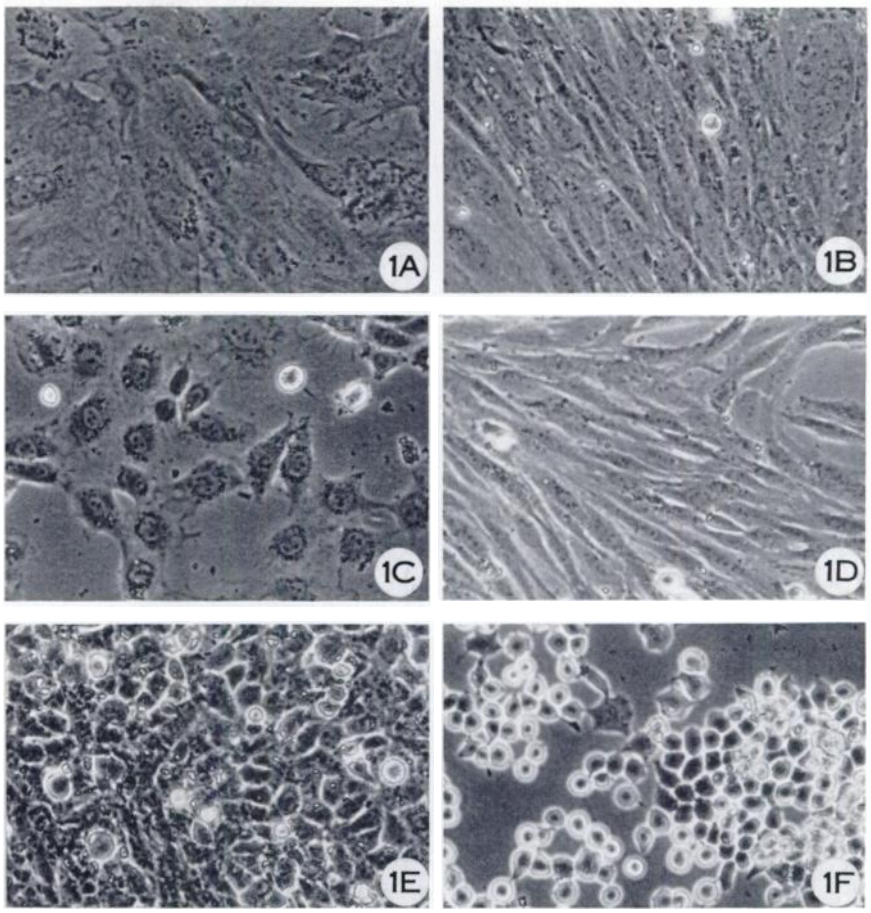

Fig. 1. Cell morphology in monolayer culture. Monolayer cultures were grown as described in "Materials and Methods." *A*, low-passage rat embryo fibroblast cells; *B*, N2 (normal rat embryo fibroblast line); *C* to *F* (cells subcloned from N2 after transformation): *C*, NG-E (MNNG transformant); *D*, SV-3 (SV40 transformant); *E*, PY-3 (PY transformant); *F*, RS-1 (RSV transformant).

Table 2

Collagen synthesis and secretion by normal and transformed rat embryo fibroblasts

		% of collagen[a]		
Transforming agent	Cell line	Cell layer	Medium	Total
None	LRE[b]	2.23	31.0	5.0
	3Y1	1.16	49.0	2.9
	N2	0.64	24.7	1.6
RSV (revertant)	RS-2	1.52	62.5	4.2
MNNG	NG-8	0.80	26.4	1.7
	NG-E	0.60	15.1	1.0
SV40	SV-1	1.31	94.8	3.6
	SV-3	0.72	31.1	1.2
PY	PY-2P2	0.62	16.1	1.4
	PY-2N	0.47	13.1	0.9
	PY-2	0.43	12.7	0.9
	PY-3	0.47	7.0	0.6
RSV	RS-4N	0.31	6.7	0.4
	RS-4	0.28	4.9	0.4
	RS-1	0.18	1.1	0.2
	RS-6	0.16	1.7	0.2
	RS-5	0.18	0.9	0.2

[a] The percentage of collagen synthesis was calculated as described by Diegelmann and Peterkofsky (10). The results are the averages of 2 experiments that agreed closely.

[b] LRE, low-passage rat embryo fibroblasts.

of normal progenitor cell levels of collagen. The RSV transformants, which were more transformed by the criteria of morphology and tumorigenicity, synthesized even less collagen, between 11 and 25% of control cell levels.

Proline-specific Activity. Although PY and RSV lines synthesized a reduced percentage of collagen, relative to N2 cells, both types of transformants also incorporated approximately 2-fold more proline per μg protein. Thus, if collagen synthesis is expressed in cpm/μg protein, the reduction in collagen synthesis in the transformants is less. [^{3}H]Proline incorporation by transformants relative to N2 was the same at 4 or 8 hr. Therefore, the difference in incorporation probably did not result from differences in proline transport. On the other hand, specific activity of transformed *versus* normal cell proteins was shown to be increased by 2 methods, comparison of Coomassie blue staining and autoradiographic exposure of a protein band on an acrylamide gel and measurement of specific activity of proline and hydroxyproline in hydrolysates of culture medium proteins.

If the collagenous [^{3}H]proline cpm synthesized per μg cell protein was corrected for the increased specific activity of the protein synthesized by selected transformants, it is clear that the decrease in collagen synthesis is an absolute decrease. When specific activity determined by the gel scan is used in this correction, PY-3 cells made 25% and RS-1 cells made 7% of the level of collagen per μg total protein that the N2 cells made. If the specific activity determined by amino acid analysis was used, the PY-3 and RS-1 cells made 51 and 12%, respectively, of the collagenous protein per μg of cell protein that the N2 cells made.

Effect of Exogenous Proline on Collagen Synthesis. One possible explanation for the increased specific activity of proline in proteins synthesized by transformed cells is higher

specific activity of intracellular proline pools resulting from reduced endogenous proline synthesis. If this is the case, decreased endogenous proline pools might limit collagen synthesis, since collagen has a 5-fold-higher level of proline than most proteins. Alternatively, proline synthesis and consequently pool size could have been reduced by some feedback mechanism after collagen synthesis was decreased. In order to determine if the size of proline pools limited collagen synthesis, N2, PY-3, and RS-1 cells were grown in the presence of 0, 0.1, 0.5, 1.0, 5, and 10 mM proline for 2 days. Cultures were pulsed with [^{3}H]alanine for 30 min at the end of this period, and collagen synthesis was measured by collagenase assay. Exogenous proline had no effect on the percentage of collagen synthesized by normal or transformed cultures (data not shown).

Mechanism of the Decrease in Collagen Synthesis in Transformed Cells. Reduced collagen synthesis has been observed in a variety of transformed cells relative to their normal counterparts. This decrease has been correlated with reduced levels of procollagen mRNA in RSV-infected chicken embryo fibroblasts (1, 29, 31). Our findings and those of Hata and Peterkofsky (15) that different transforming agents have different effects on collagen synthesis raised the question of whether transforming agents other than RSV and agents in cells other than chick cells also affect the level of procollagen mRNA. We isolated total RNA from 2 normal cell types (3Y1 and N2) and from one or more of each of the groups of lines clonally derived after treatment with one of the 4 transforming agents, MNNG (NG-E), SV40 (SV-3), PY (PY-2P2, PY-2N, and PY-3), and RSV (RS-1 and RS-4N). The RNA's were translated *in vitro* in a reticulocyte lysate system, which was optimized for procollagen synthesis (29). Immunoprecipitable procollagen synthesis (Chart 2) was linear with RNA input up to 8 μg/ml of reaction, and all RNA samples stimulated similar levels of total incorporation. Immunoprecipitation, with anti-rat type I procollagen serum, of products from the *in vitro* translation of different RNA's indicated significant differences in procollagen synthesis. The RNA's from 3Y1, N2, and NG-E cells (Chart 2, *A* and *B*) all stimulated significant procollagen synthesis; N2 and NG-E preparations, however, stimulated only about 70% of the synthesis stimulated by RNA from the 3Y1 cells. SV40, PY, and RSV RNA preparations stimulated less procollagen synthesis. Of these, the transformant whose RNA stimulated the most procollagen synthesis, SV-3 (Chart 2*B*), stimulated only about 50% of N2 levels of type I procollagen translation. PY-2P2 and PY-2N RNA's (Chart 2*C*) synthesized only 23 and 17%, respectively, of 3Y1 levels of *in vitro* collagen synthesis. PY-3, RS-4N, and RS-1 (Chart 2, *C* and *D*) all synthesized low levels of procollagen *in vitro*, about 21% of N2 RNA levels of type I procollagen synthesis. These comparisons were all made at 8 μg of RNA per ml of reaction.

Although the transformed cell lines fall into the same general order of relative synthesis, there are significant differences between the levels of transformed cell collagen synthesis relative to the normal progenitor collagen synthesis when levels of synthesis are determined by *in vitro* translation rather than *in vivo* labeling. It is not clear whether this is a function of variation among transformants in the types of collagen synthesized in tissue culture, which are not monitored in our translation assay (optimized for type I and immunoprecipitated by anti-type I serum), due to changes in translational control or

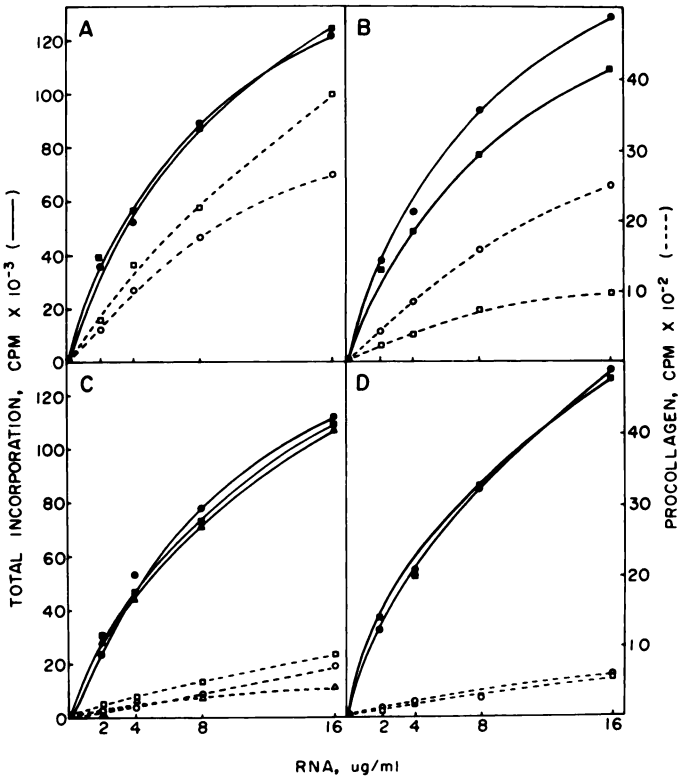

Chart 2. Immunoprecipitation of type I procollagen from *in vitro* translation products of rat embryo fibroblast RNA. RNA was isolated from rat embryo fibroblasts, and 0.2, 0.4, 0.8, and 1.6 μg were used to prime 100-μl reticulocyte lysate translation reactions in the presence of [^{3}H]proline as described (29). Three μl of rabbit anti-rat type I procollagen serum were reacted with 50 μl of lysate, and the mixture was precipitated with excess sheep anti-rabbit IgG. Backgrounds, determined by immunoprecipitation of translation products with preimmune serum, were subtracted. Totals were determined by precipitation of 5 μl of the mixture onto Whatman 540 filters in 10% trichloroacetic acid as described (29). ■, ●, ▲, total incorporation; □, ○, △, anti-rat type I procollagen immunoprecipitation; *A*: □– – –□, ■——■, 3Y1; ○– – –○, ●——●, N2; *B*: ○– – –○, ●——●, NG-E; □– – –□, ■——■, SV-3; *C*: □– – –□, ■——■, PY-2N; ○– – –○, ●——●, PY-2P2; △– – –△, ▲——▲, PY-3; *D*: □– – –□, ■——■, RS-4N; ○– – –○, ●——●, RS-1.

differences in turnover. In order to determine whether a major collagenous component was synthesized in our *in vitro* system but not immunoprecipitated, translation products were electrophoresed on SDS-polyacrylamide gels as described (Fig. 3). In all cases except the RSV transformants, the major high-molecular-weight [^{3}H]proline-labeled proteins comigrated with type I procollagen standards.

Unusual Collagen Processing in RS-1 Cells. Although the level of collagen synthesized by RS-1 cells is greatly reduced, collagenase assays and gel electrophoresis of medium proteins indicated that there was some collagen synthesis. We were interested in determining whether this was a different type of collagen, which was resistant to the effects of transformation, or whether it represented low-level synthesis of the type I procollagen which we observed in the normal cells and in the other transformants. If equal amounts of radioactivity of normal and RS-1 cell medium proteins were electrophoresed and compared, RS-1 preparations contained a band migrating slightly slower than proα1(I) and a band migrating slightly slower than proα2 (data not shown). In order to determine whether these band pairs were related to proα1(III) and $P_N\alpha$1(III) or proα1(I) and proα2(I), medium and cell layer proteins were

pepsin digested and electrophoresed under nonreducing conditions (Fig. 4) (33). Because pepsin-digested type III collagen contains interchain disulfide bonds and type I does not, unreduced type III collagen migrates only slowly into the gel and consequently can be readily distinguished from type I or type I trimer collagens which do not contain interchain disulfide bonds and therefore migrate as single chains after pepsin digestion.

The bands, migrating in approximately the position of α1(I) and α2(I), constituted the major pepsin-resistant proteins in unreduced gel patterns of RS-1 cell layer and medium (Fig. 4). The ratio of α1 to α2 bands measured by densitometric scanning in their linear range of exposure was about 3 and was not significantly different from the corresponding ratio in N2 protein gels. Both the α1 and α2 bands migrated slightly slower than their N2 counterparts. To determine whether this was attributable to differences in the extent of hydroxylation, which have been reported among different cell lines, we labeled N2 and RS-1 cultures in the presence of α,α'-dipyridyl (9), an inhibitor of prolyl and lysyl hydroxylases, and processed the proteins as described above. Samples were maintained at 4° at all times in order to avoid unfolding of the collagen helix. In Fig. 4, the pepsin-resistant proteins from untreated (*Lanes 1* and *2*) and treated (*Lanes 3* and *4*) N2 (*Lanes 1* and *3*) and RS-1 (*Lanes 2* and *4*) cell layers are shown after gel electrophoresis. α,α'-Dipyridyl treatment increased the migration of discrete α1 and α2 bands in both N2 and RS-1 products, indicating that hydroxylation was inhibited. The bands representing α1 and α2 chains in the treated RS-1 sample (*Lane 4*) migrated more similarly to the corresponding bands in the N2 sample (*Lane 3*) than was the case in the absence of the inhibitor (*Lanes 1* and *2*). It seems probable that the RS-1 collagen represents a modified form of type I collagen which migrates more slowly in SDS-polyacrylamide gel electrophoresis at least partially because of increased hydroxylation and/or glycosylation.

DISCUSSION

Reduced collagen synthesis was first associated with SV40 transformation of 3T3 cells in 1966 (13); since that time, investigators have reported that collagen synthesis is reduced in 3T3 cells transformed by sarcoma viruses and 4-nitroquinoline-1-oxide, a chemical carcinogen (15), chicken embryo fibroblasts transformed by RSV (2, 20, 23, 29, 31), and mouse mammary tumor cells (28). Nonetheless, the relationship of transformation to collagen synthesis has remained undefined, and the molecular mechanism of the decrease in collagen synthesis has been investigated only in RSV-transformed chicken embryo fibroblasts (1, 29, 31). We have made use of a series of normal rat embryo fibroblast lines and their transformed counterparts to study the effect of different transforming agents on collagen synthesis by related cells and to determine the degree of correlation between collagen synthesis levels and more classical measures of transformation.

We report here on our studies of collagen synthesis in 2 related clonally derived normal rat embryo fibroblast lines and 13 subclones, each selected after transformation with one of 4 transforming agents, MNNG, SV40, PY, and RSV. Each agent produced transformants with similar levels of collagen synthesis, and the extent of the decrease in synthesis, when it occurred, correlated qualitatively with the extent of transformation as defined by morphology, saturation density, growth in agarose, and tumorigenicity. We also observed decreasing levels of collagen synthesis with increasing culture time in studies of early-passage rat fibroblast clones (3Y1 and N2) carried in culture for different periods of time. The level of collagen synthesis by transformants appeared to be a function of both the original level of collagen synthesis in the cells from which transformants were derived and the transforming agent.

A low-passage rat fibroblast strain, 3Y1, the original normal clone, and N2, a subclone of 3Y1, synthesized the highest levels of collagen among the cells tested. Type I procollagen mRNA activity in the normal clones correlated with relative levels of collagen synthesis. The 2 MNNG transformants had almost the same levels of collagen synthesis as did the normal progenitor N2 clone (Table 2) and were also the least transformed by the other criteria. Of 5 mice given injections of MNNG transformants, none developed tumors. Procollagen mRNA activity was not affected significantly in the one MNNG transformant examined.

SV40 transformants were slightly more transformed than were MNNG transformants and did cause tumors when injected into nude mice. The 2 SV40-transformed lines were only mildly affected in collagen synthesis relative to progenitor cells. *In vitro* translation of RNA isolated from one of them, SV-3, surprisingly resulted in significantly reduced levels of type I procollagen synthesis relative to N2 RNA (Chart 2).

The PY transformants were significantly transformed by all criteria and were also significantly reduced in collagen synthesis and procollagen mRNA activity. They synthesized between 30 and 48% of normal progenitor collagen synthesis. As in the case of the SV40 transformant, procollagen mRNA activity, which was 17 to 23% of normal progenitor levels, was more reduced than was collagen synthesis. The reason for the differences between collagen synthesis measurements and procollagen mRNA activity is not clear, but several possibilities are discussed below.

The RSV transformants of N2 were significantly transformed by all criteria; they were more transformed morphologically and caused tumors which grew faster than the tumors caused by PY transformants. They were also most reduced in collagen synthesis, which ranged from 12 to 25% of N2 controls. Procollagen mRNA activity in these cells was similar to the lowest of the 3 PY transformants examined and was 21% of N2 levels. RS-1 transformants made a low level of type I collagen which migrated more slowly than N2 collagen, at least in part as a result of increased hydroxylation and/or glycosylation.

Interestingly, nude mice given injections of the RSV transformants developed unusual tumors which seemed to have minimal connective tissue matrices and were highly vascularized in contrast to the solid encapsulated tumors formed by SV40 and PY cells. These mice also showed early signs of hemorrhage at the site of injection.[6] These observations suggest that the RSV transformants may cause tumors which contain reduced levels of collagen in the tumor stroma. There is some controversy concerning the source of collagenous tumor stroma, *i.e.*, whether it is synthesized by recruited normal cells or by the tumor cells. Our observations are consistent

[6] S. Sandmeyer, R. Smith, D. Kiehn, and P. Bornstein, unpublished observations.

with those of Roesel *et al.* (28) who concluded that tumor cells synthesized levels of collagen that were similar to their tissue culture counterparts. Such cells may therefore be responsible for synthesis of at least some of the collagenous tumor matrix.

In RSV-transformed chicken embryo fibroblasts, the reduction in collagen synthesis is correlated with a decrease in transcription of the type I procollagen genes[7] and a resulting decrease in the level of procollagen mRNA (1, 29, 31). We have investigated the activity of type I procollagen mRNA in 7 transformants. In 6 of these, representing transformation by SV40, PY, and RSV, transformation was accompanied by a decrease in type I procollagen mRNA activity. The extent of the decrease in mRNA activity correlated generally with the decrease in the level of collagen synthesis and the degree of transformation as determined by several other criteria. However, the reduction in mRNA activity, when it occurred, did not always correlate quantitatively with the observed level of collagen synthesis in cell culture.

The effect of transformation on SV40 and PY transformant type I procollagen mRNA activity was greater than the effect on collagen synthesis. In one MNNG and one RSV transformant, the decrease in mRNA levels was somewhat less than the decrease in collagen synthesis. These differences between type I mRNA activity and total collagen synthesis in transformed relative to normal cells could be attributed to differences in the types of collagen synthesized, translational regulation, or stability of the collagens. Differences in the types of collagen synthesized with transformation have been observed by other investigators (15). However, in our studies in gel electrophoresis of medium, cell layer, and *in vitro* translation products of normal and transformed cells, type I procollagen was the predominant species with the possible exception of the RSV transformants. Translational regulation could explain increases or decreases in collagen synthesis relative to type I procollagen mRNA activity. This possibility could be evaluated by quantitating type I collagen synthesized in culture and comparing levels of synthesis with levels of type I procollagen mRNA determined by translation or hybridization assay.

Another significant factor in the level of collagen synthesized by fibroblasts is intracellular helix stability. Bienkowski *et al.* (5) have reported that 30% of collagen synthesized by exponentially growing human fibroblasts in culture is degraded intracellularly. It is thought that this collagen is underhydroxylated and is degraded because of an unstable structure. Synthesis decreases with increasing cAMP concentrations in the cell (4), and this decrease appears to be related to an increase in collagen degradation (3). Levinson *et al.* (23) have reported that hydroxylase activity is significantly increased in transformed cells, which are also known to have depressed levels of cAMP (32). It would be interesting if these factors combined to produce more efficient collagen synthesis despite a reduction in procollagen mRNA levels in these cells.

There is an increasing amount of information available on changes in the collagen-synthetic apparatus after transformation. We report here that collagen synthesis and type I procollagen mRNA activity generally decrease in parallel with increasing transformation. In 2 transformants, our data suggest that endogenous proline pools are affected by transformation. The total cellular level of collagen synthesis represents a delicate balance of mRNA levels, translational efficiency, processing, and degradation. Transformation is a complex phenomenon which may impinge on several of these steps, or, alternatively, it may affect only one, precipitating a compensatory response by cellular regulatory mechanisms. Understanding the nature of these events will, in either case, greatly expand our knowledge of the regulation of collagen biosynthesis.

[7] S. Sandmeyer, B. Gallis, and P. Bornstein. Coordinate transcriptional regulation of type I procollagen genes by Rous sarcoma virus, submitted for publication.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Eva Marie Click. We also thank Dr. Byron Gallis for helpful discussions and advice.

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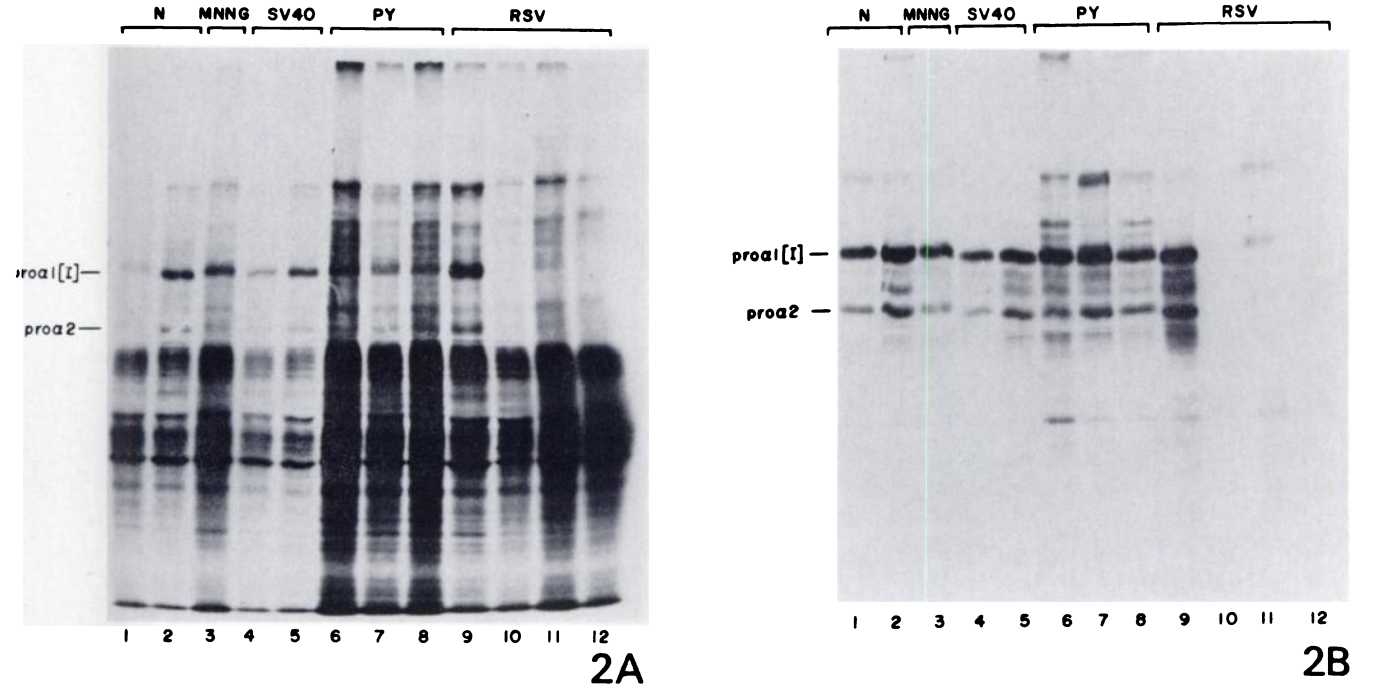

Fig. 2. Cell layer and medium proteins synthesized by normal and transformed rat embryo fibroblast lines. Nearly confluent cultures of rat embryo fibroblasts were preincubated for 1 hr in DMEM supplemented with penicillin, streptomycin, and ascorbate. Cells were labeled for 4 hr in the above medium containing 25 μCi L-[2,3-^{3}H]proline per ml. At the end of this time, medium was centrifuged briefly to remove cellular debris, and the cell layer was washed 3 times in phosphate-buffered saline and solubilized in 0.5 M ammonium hydroxide. Medium and cell layer proteins were precipitated in 10% trichloroacetic acid, washed in ethanol and ethanol-ether, and taken up in sample buffer with 50 mM dithiothreitol. Samples were boiled for 3 min and electrophoresed at 30 mamp in a 5% SDS-polyacrylamide gel with a 2.5% stacking gel, and the gels were processed for fluorography. The radioactivity in each *lane* represents 5 × 10^4 cells or the medium equivalent. *A*, cell layer protein; *B*, medium protein; *Lane 1*, N2; *Lane 2*, 3Y1; *Lane 3*, NG-8; *Lane 4*, SV-3; *Lane 5*, SV1; *Lane 6*, PY-2N; *Lane 7*, PY-2P2; *Lane 8*, PY-2; *Lane 9*, RS-2; *Lane 10*, RS-6; *Lane 11*, RS-4; *Lane 12*, RS-5.

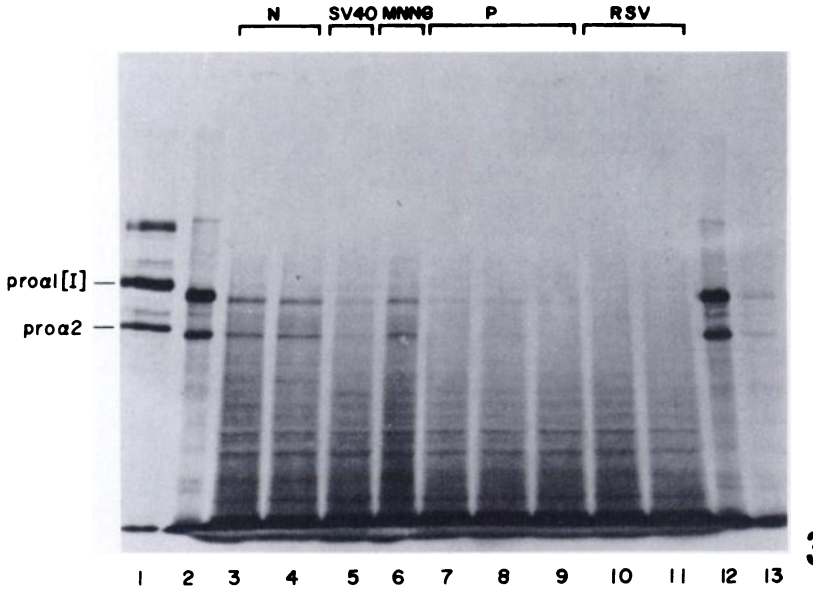

Fig. 3. SDS-polyacrylamide gel electrophoresis of *in vitro* translation products of normal and transformed rat embryo fibroblast RNA. Reticulocyte lysate reactions were primed with 0.8 μg total RNA per 50-μl reaction in the presence of [^{3}H]proline as described (29). Twenty-five μl of reaction mix were supplemented with 10 μl of 3× the sample buffer of Laemmli (22), brought to 100 mM dithiothreitol, boiled for 3 min, and electrophoresed directly on a 5% polyacrylamide gel with a 2.5% stacking gel at 30 mamp. *Lane 1*, N2 medium; *Lane 2*, procollagen immunoprecipitated from [^{3}H]-proline-labeled cell layer incubated with 15.5 μg of α,α′-dipyridyl per ml as described in "Materials and Methods;" *Lanes 3 to 11* (*in vitro* translation mixtures primed by rat cell RNA): *Lane 3*, N2; *Lane 4*, 3Y1; *Lane 5*, SV-3; *Lane 6*, NG-E; *Lane 7*, PY-2N; *Lane 8*, PY-2P2; *Lane 9*, PY-3; *Lane 10*, RS-4N; *Lane 11*, RS-1; *Lane 12*, same as *Lane 2*; *Lane 13*, same as *Lane 2*, except that preimmune serum was used.

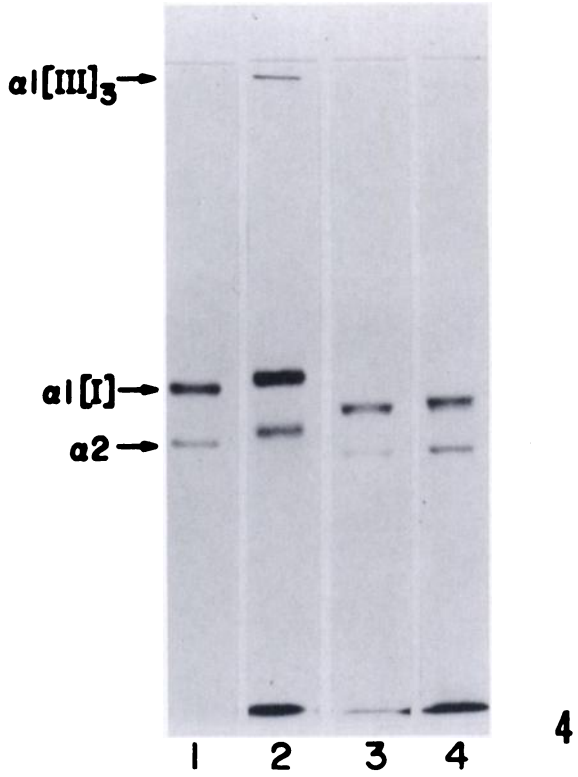

Fig. 4. Gel electrophoresis of pepsin-digested N2 and RS-1 cell layer and medium protein synthesized in the presence and absence of α,α′-dipyridyl. N2 cells and RS-1 cells were labeled for 6 hr with 50 μCi of [2,3-^{3}H]proline per ml in DMEM supplemented with penicillin and streptomycin and 15.5 μg of α,α′-dipyridyl per ml. Cell layer proteins were digested and electrophoresed as described in the legend to Fig. 3 except that samples were not reduced. *Lanes 1* and *3*, normal cell layers; *Lanes 2* and *4*, RS-1 cell layers; *Lanes 1* and *2*, minus α,α′-dipyridyl; *Lanes 3* and *4*, plus α,α′-dipyridyl.

Correlation of Collagen Synthesis and Procollagen Messenger RNA Levels with Transformation in Rat Embryo Fibroblasts

Suzanne Sandmeyer, Ronald Smith, Donald Kiehn, et al.

Cancer Res 1981;41:830-838.