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Mechanisms of Kirsten murine sarcoma virus transformation-induced changes in the collagen phenotype and synthetic rate of BALB 3T3 cells

(productive infection and transformation/temperature-sensitive Kirsten sarcoma virus/procollagen mRNA)

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ABSTRACT **Specific viral transformation rather than cell selection can explain the previously observed increase in the proportion of type III procollagen compared to type I procollagen in BALB 3T3 cells transformed by Kirsten murine sarcoma virus (Ki-MSV). Two subclones of BALB 3T3 A31 were productively infected with a temperature-sensitive Ki-MSV in the presence of helper murine leukemia virus (MLV), resulting in virtually complete transformation of cultures and eliminating selection of transformed foci. Analysis of radioactive collagen, derived from procollagen by pepsin treatment, showed that both of the tsKi-MSV/MLV-transformed subclones contained a 4-fold greater proportion of type III procollagen than did control MLV-infected cultures. A nonproducer derivative exhibited an even greater change (10-fold), indicating that viral replication was irrelevant. After 48 hr at a nonpermissive temperature, tsKi-MSV-transformed cells retained a high proportion of type III procollagen, suggesting that either this change is not induced by *src* protein or else there is a slowly reversible or irreversible step involved. Alternatively, type III procollagen mRNA may be long lived. In contrast, the relative rate of procollagen synthesis in transformed cells was clearly regulated by *src* protein. Translation of mRNA from cells preincubated at permissive or nonpermissive temperatures revealed that the decreased relative rate can be explained by a simultaneous small decrease in the level of procollagen mRNA and a large increase in mRNA for noncollagen proteins.**

Transformation of cells by tumor viruses results in decreased anchorage- and density-dependent growth, changes in cellular morphology (1), and generally a reduction in synthesis of extracellular proteins such as fibronectin (2) and procollagen (3–6). In fibroblasts transformed by Rous sarcoma virus (RSV), these decreases correlate with a reduction in procollagen and fibronectin mRNA (7–9). 3T3 mouse cells transformed by murine sarcoma virus (MSV) also were reported to have a decreased level of procollagen mRNA hybridizable to a cDNA probe (10).

There are a number of genetically distinct collagen types, the properties of which have been reviewed in detail (11, 12). Type I contains α_1(I) and α_2 subunits in a 2:1 ratio as a trimer; cartilage-specific type II is a homotrimer of α_1(II); and type III, $[\alpha_1(III)]_3$, uniquely has intrahelical disulfide bonds. Type IV is a basement-membrane collagen, and type V or AB collagen appears to be associated with the basement membrane in some tissues. A previous report from this laboratory (5) demonstrated that BALB 3T3 A31 subclones synthesized mainly type I collagen, but established Kirsten (Ki) or Moloney MSV-transformed 3T3 lines also produced significant amounts of type III. The virally transformed cell lines used were nonproducers selected as foci at a relatively low frequency (13). Because BALB 3T3 A31 cultures are quite heterogeneous, clones selected on the basis of their transformation might have been derived from variant cells already producing type III collagen. The studies presented here were directed at determining whether cell selection or viral transformation was responsible for the altered collagen phenotype.

The collagen produced by two stable, nontransformed subclones was examined before and after the direct productive infection, and transformation of these cultures with Ki-MSV in the presence of helper murine leukemia virus (MLV), which allows replication of the defective MSV. Comparisons were made with control cultures infected with MLV alone. MSV temperature sensitive for transformation was used in order to determine the role of the *src* gene product. Results demonstrated that MSV transformation, and not merely infection with MLV, induced an increase in the proportion of type III collagen produced. Further experiments were conducted to determine the mechanism of this change.

MATERIALS AND METHODS

Cell Cultures. Subclones 3T3-P3 and 3T3-714 were derived from BALB 3T3 A31 cells (14) and described previously (5, 15). Both subclones exhibit density-dependent growth, do not grow in soft agar, and are flat with polygonal shapes. They were infected with a stock of temperature-sensitive Ki-MSV (ts371) containing helper Moloney MLV as described by Shih *et al.* (16). The transformation was kindly carried out by E. Scolnick and R. Goldberg. The resultant transformed cultures of 3T3-P3 and 3T3-714 were designated tsKi(MLV)-3T3-P3 and tsKi(MLV)-3T3-714. These cells were temperature sensitive with respect to transformed morphology and growth in soft agar and produced MSV, as evidenced by a high level of reverse transcriptase (RNA-dependent DNA polymerase) in the medium and positive focus formation against monolayers of 3T3-714. A subclones, designated tsKi-3T3-714, was selected from tsKi(MLV)-3T3-714 growing in soft agar and was defective with respect to virus production but exhibited temperature-sensitive morphology and growth in soft agar. Control cultures infected with MLV alone remained untransformed and were designated (MLV)-3T3-P3 or -714. A nonproducer transformant (Ki-3T3-234), had been used previously (5). Cells were propagated at 36°C, permissive for the ts lines, as described previously (17).

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Abbreviations: RSV, Rous sarcoma virus; MSV, murine sarcoma virus; Ki-MSV, Kirsten MSV; MLV, murine leukemia virus; ts-, temperature sensitive.

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To test temperature sensitivity, cultures were transferred to an incubator at 40°C under 95% air/5% CO_2.

Growth in Soft Agar. Growth in 0.5% agar was determined as described (18). Cells were grown at 36°C or 40°C for 24 or 48 hr, trypsinized, seeded at 3×10^4 per dish, and incubated at either 36°C or 40°C for 8 days, at which time Polaroid photographs of the dishes were taken. The number of large colonies in the field was determined and compared to the total number of cells visible.

Incubation with Radioactive Proline. Growth medium from late-logarithmic-phase cells was replaced with serum-free minimal essential medium containing 0.1 mM sodium ascorbate and 0.1 mM β-aminopropionitrile. Cells were labeled at 36°C or 40°C with 5 μCi (1 Ci 3.7×10^{10} becquerels) of L-[^{14}C]proline (New England Nuclear, final concentration 0.1 mM) as described (5, 17). Cell and medium fractions containing radioactive procollagen were prepared as previously described (17), except that the medium was dialyzed exhaustively against 0.5 M acetic acid. This step and subsequent steps were performed at 4°C. The dialyzed medium was lyophilized and redissolved in 0.5 M acetic acid. The saline-washed cell pellet was resuspended in 0.05 M Tris·HCl, pH 7.6, sonicated, and dialyzed against 0.5 M acetic acid. The retentate was lyophilized and treated in the same manner as the medium fraction. Portions of the medium and sonicate were assayed for procollagen content as described below. To simplify interpretation of data obtained from $NaDodSO_4$/polyacrylamide gel electrophoresis, procollagen in the fractions was converted to collagen by removing propeptides with limited pepsin digestion at a final protein-to-pepsin ratio of 6:1 (100–200 μg/ml) at 15°C for 6 hr. The helical collagen region is resistant to proteolysis at this temperature. Protein content was determined with Bio-Rad protein reagent (19), using gamma globulin standard. There was no preferential degradation of α_1(III) chains, which may occur under more vigorous conditions (20). The pepsin digest was clarified by centrifugation (10,000 × g, 15 min), and the supernatant solution was lyophilized and prepared for gel electrophoresis as described below.

Assay of Extracellular Procollagen Degradation. Types I and III procollagens from the medium of Ki-3T3-234 cells labeled with [^{14}C]proline for 16 hr were purified by ammonium sulfate precipitation (5). Logarithmic-phase cells were preincubated as described above, 6×10^4 dpm of [^{14}C]proline-labeled Ki-3T3 procollagen was added, and incubation was continued for 16 hr. After incubation, the medium was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis as outlined above.

RNA Extraction and Cell-Free Translation of mRNA. Total cellular RNA was extracted from late-logarithmic-phase cells by the guanidine procedure (7, 21). RNA concentration was measured by absorbance at 260 nm, and portions were stored in liquid N_2. Nuclease-treated rabbit reticulocyte lysate (22) was supplied by Amersham. Translation was performed in a reaction mixture consisting of 8 μl of lysate, 10 μCi of L-[2,3,4,5-^{3}H]proline (120 Ci/mmol, Amersham), 0.15 M potassium acetate, and 1 μl (2 μg) of total RNA, denatured at 70°C for 1 min before addition (23), in a final volume of 11 μl. Duplicate tubes for each RNA sample were prepared and incubated for 2 hr at 26°C. Twenty microliters of 1 M Tris·HCl, pH 10/5 mM L-proline was added, and incubation was continued for 30 min to deacylate tRNA. The samples were assayed for procollagen synthesis as described below.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Electrophoresis was performed at 50 mA for 3 hr on 5% slab gels with 3.5% stacking gels (24), with 2 M urea in the gel to aid in separating α_1(I) and reduced α_1(III) chains (25), but the degree of separation varied. Lyophilized samples were dissolved in 0.05 M Tris·HCl, pH 7.6, and 25-μl replicate portions were incubated for 60 min at 37°C with 2 μl of 31 mM *N*-ethylmaleimide/6 mM $CaCl_2$ either with or without 0.3–0.4 μg of purified bacterial collagenase. Sample buffer (20% sucrose/4% $NaDodSO_4$/1 M urea/0.002% bromophenol blue), 25 μl, was added, and samples were denatured at 70°C for 15 min. Some samples were reduced with 10 mM dithiothreitol prior to denaturation. Because type III, but not type I, collagen contains intrahelical disulfide bonds, it migrates as a trimer in the absence of dithiothreitol but is dissociated in its presence to α_1(III) chains, which migrate slightly slower than α_1(I) chains. Gels were calibrated by using [^{14}C]proline-labeled acid-soluble collagen (26). Bands were visualized by fluorography (27) and quantitated by densitometry after preexposure of the x-ray film to light, to obtain linearity between image intensity and radioactivity (28). Proportions of peaks on scans were determined by planimetry.

Quantitative Assay of Collagen by Collagenase Digestion. Collagen in 10% trichloroacetic acid-precipitated proteins was measured with purified bacterial collagenase as described (29, 30). The relative rate of procollagen synthesis as a percentage was calculated by using the formula [1.13(collagen dpm)] × 100/{4.3[(noncollagen protein dpm − 0.13(collagen dpm)] + 1.13(collagen dpm)}, which corrects for the higher imino acid content of procollagen relative to noncollagen protein and for the failure of collagenase to digest most of the procollagen extension peptides (31).

RESULTS

Effect of Productive Infection and Transformation by Ki-MSV/MLV on Procollagen Synthesis and Phenotype. To determine whether cell selection led to the previously observed altered collagen phenotype, subclones 3T3-P3 and 3T3-714 were productively infected with tsKi-MSV and helper MLV. No subcloning was necessary because the tsKi-MSV replicated and continuously infected and transformed the entire culture. In addition to the characteristic changes in cellular properties, MSV transformation of both subclones led to a 50% decrease in the relative rate of procollagen synthesis at the permissive temperature, levels similar to those of established lines of nonproducer transformants (5). This decrease was temperature sensitive (data not shown).

Collagen from the medium of either infected or infected/transformed P3 and 714 subclones, labeled with [^{14}C]proline at the permissive temperature, was examined by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1). In other experiments, the proportion of collagen chains in the medium after a 16-hr labeling period was quantitated by scanning fluorograms (Table 1). The media of both control cultures, (MLV)-3T3-P3 (Fig. 1, lanes 1–3) and (MLV)-3T3-714 (Fig. 1, lanes 7–9), contained type I collagen almost exclusively, because α_1(I) and α_2 chains were present in the ratio of approximately 2:1. A faint band appeared at the position of collagen trimer molecules (Fig. 1, lanes 1 and 7). After reduction, the band disappeared and a band migrating just above α_1(I) appeared (Fig. 1, lanes 2 and 8), behavior consistent with that of α_1(III) chains (25). This component was further identified by delayed reduction electrophoresis (32), in which the trimer is dissociated after electrophoresis is in progress, resulting in decreased mobility of α_1(III) chains (data not shown). Type III collagen represented approximately 5–6% of the total radioactive collagen in the medium of nontransformed, MLV-infected cells (Table 1).

Bands corresponding to type III collagen were more intense in samples from the infected/transformed cells, tsKi(MLV)-3T3-P3 (Fig. 1, lanes 4–6) and tsKi(MLV)-3T3-714 (Fig. 1, lanes

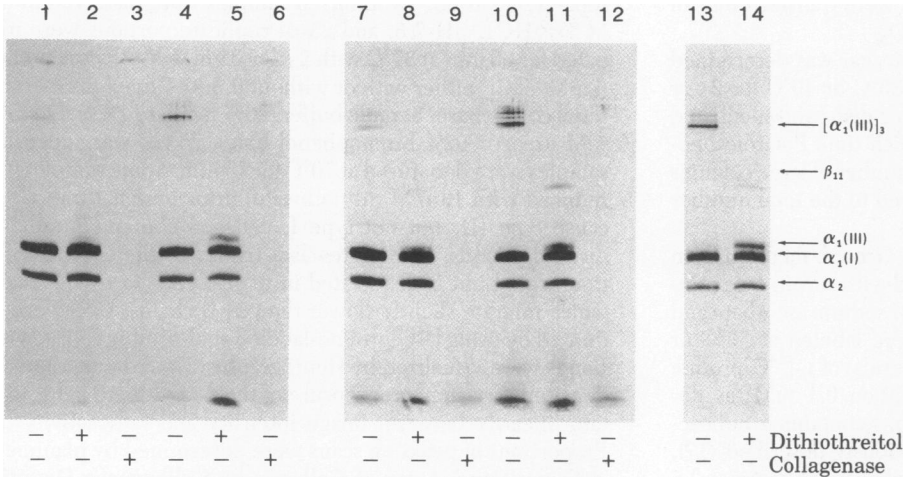

FIG. 1. Collagen chains synthesized by 3T3 subclones infected by MLV or infected and transformed by MLV/tsKi-MSV. Cultures were labeled with [^{14}C]proline for either 3 hr [MLV-3T3-P3, lanes 1–3, and tsKi(MLV)-3T3-P3, lanes 4–6] or 16 hr [MLV-3T3-714, lanes 7–9, and tsKi(MLV)-3T3-714, lanes 10–12] at 36°C and collagen from the medium was analyzed on gels and fluorograms. Lanes 13 and 14 are collagen from Ki-3T3-234 (5). Samples indicated were pretreated with collagenase or dithiothreitol.

10–12), and quantitation showed the proportion of type III to be approximately 20% of total collagen produced (Table 1). These findings are similar to the results obtained previously with nonproducer Ki-3T3 cells (5). Although almost all of the radioactive procollagen appeared in the medium after 16 hr, a similar analysis of collagen in the cell fraction was carried out. Transformed cell fractions contained a significantly higher proportion of type III collagen compared to nontransformed cells but, as observed previously (33), the proportion was lower than in the medium fraction.

Effect of Temperature Shift on Procollagen Synthesis and Phenotype of tsKi-MSV Transformed Cells. To explore the mechanism for the Ki-MSV-provoked change in the collagen phenotype, the temperature sensitivity of the putative *src* protein (16) was exploited. A nonproducer subclone, tsKi-3T3-714, was used for these experiments because preliminary experiments showed that it exhibited a larger proportion of type III collagen and withstood exposure to 40°C better than the productively infected cultures. Its morphology at the permissive temperature was typically that of transformed cells, but when switched to the nonpermissive temperature (40°C) it regained a flat morphology by 24 hr. At 36°C, 3T3-714 did not grow in soft agar (Fig. 2*A*) whereas 80% of tsKi-3T3-714 cells in the agar formed large colonies (Fig. 2*D*), a property closely correlated with tumorigenic potential (1). After 24 hr at 40°C, this property was almost completely lost (Fig. 2*E*); even those colonies that grew had much smaller diameters than those at the permissive temperature. By 48 hr, the transformed culture (Fig. 2*F*) was virtually indistinguishable from the nontransformed culture at 40°C (Fig. 2*C*).

The effects of temperature shift on both the relative rate of procollagen synthesis and the collagen phenotype were examined by growing the parent and transformed cells at 36°C and then transferring duplicate cultures to 40°C for 24 or 48 hr. Procollagen synthesis was measured by collagenase digestion of the cell and medium fractions (Table 2), and the proportion of collagen types in the medium was quantitated (Table 2) from scans of gel fluorograms (Fig. 3). At the permissive temperature, the relative rate of procollagen synthesis was reduced to about one-third that of the parent line. By 24 hr at the nonpermissive temperature, the rate was restored to the nontransformed level. The effect on the collagen phenotype was in marked contrast to these results. Less than 4% of the collagen secreted into the medium by the parent 3T3-714 cells consisted of type III at 36°C (Fig. 3*A* and Table 2); preliminary experiments had shown that this proportion was unchanged at 40°C (data not shown). Type III collagen represented approximately 38% of the radioactive collagen of tsKi-3T3-714 grown and labeled at 36°C (Fig. 3*B* and Table 2), more than 10 times the control value. After a temperature shift to 40°C for 24 hr, however, the proportion of type III collagen was only slightly decreased (Fig. 3*C* and Table 2), and even after 48 hr (Fig. 3*D* and Table 2) it remained almost 6-fold greater than in the parent cell collagen. The productively infected/transformed line tsKi(MLV)-3T3-P3 showed similar results; after a 20-hr shift to 40°C, the transformed cells exhibited 15% type III collagen, compared to 14% at 36°C (data not shown).

Assay for Extracellular Degradation of Types I and III Procollagens. The proportion of type III collagen could increase upon transformation by induction of type I procollagen degradation. The composition of a mixture of purified, radioactive procollagens I (77%) and III (23%) incubated with unlabeled cultures of (MLV)-3T3-P3 and tsKi(MLV)-3T3-P3 cells as above,

Table 1. Proportion of type III relative to total collagen in the medium of productively infected/transformed 3T3 clones P3 and 714

Cells	Proportion of type III collagen, %
(MLV)3T3-P3	5.9
tsKi(MLV)3T3-P3	23.2
(MLV)3T3-714	5.2
tsKi(MLV)3T3-714	19.9

Cells were labeled at 36°C for 16 hr, collagen from the medium was electrophoresed, and proportions were determined from fluorograms of unreduced samples. Procollagen secretion was approximately 90% for MLV-infected and 70% for infected/transformed cultures.

Table 2. Effect of shift to nonpermissive temperature on procollagen synthesis and phenotype of 3T3-714 and tsKi-3T3-714 cells

Cells	Preincubation time at 40°C, hr	Relative rate of procollagen synthesis, %	Proportion of type III collagen, %
3T3-714	0	3.28	3.6
	24	3.39	—
	48	3.34	—
tsKi-3T3-714	0	1.17	37.7
	24	3.13	27.7
	48	3.07	23.6

Results are derived from the experiment described in Fig. 3. The proportion of type III collagen was determined by planimetry of the scans in Fig. 3.

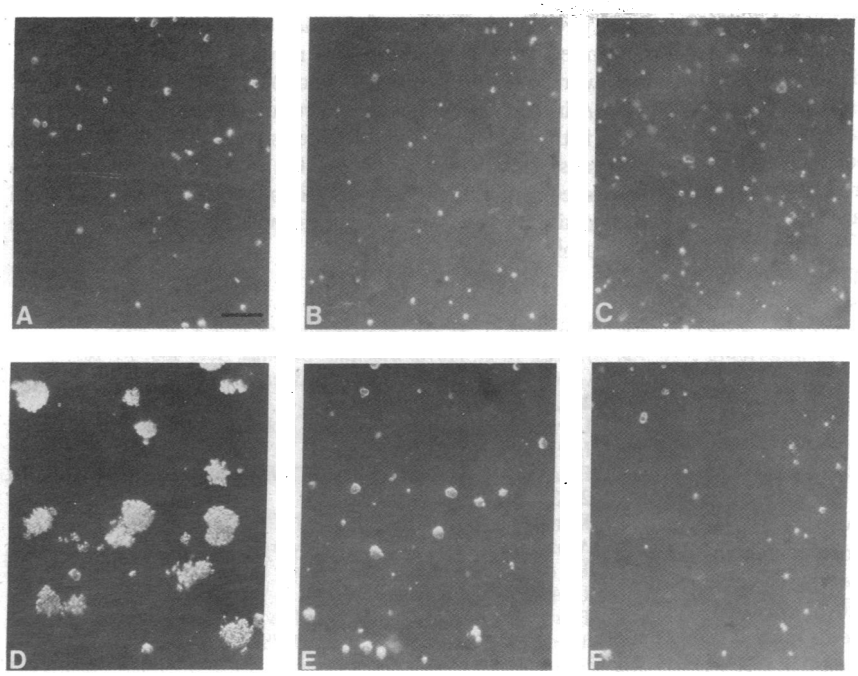

FIG. 2. Growth of control and tsKi-MSV-transformed 3T3-714 cells in soft agar at nonpermissive and permissive temperatures. Cultures were either grown at 36°C or shifted to 40°C before seeding in soft agar. 3T3-714: 36°C (*A*), 40°C for 24 hr (*B*), 40°C for 48 hr (*C*). tsKi-3T3-714: 36°C (*D*), 40°C for 24 hr (*E*), 40°C for 48 hr (*F*). The bar indicates 0.25 mm.

however, did not change (data not shown).

Cell-Free Translation of Total Cellular RNA. We examined the possibility that the Ki-MSV-induced decrease in the relative rate of procollagen synthesis resulted from a decreased amount of procollagen mRNA. (MLV)-3T3-P3 and tsKi(MLV)-3T3-P3 cells were either grown at the permissive temperature (36°C) or shifted to the nonpermissive temperature (40°C) 20 hr prior to extraction of total RNA. RNA was translated in a rabbit reticulocyte lysate system in which there was no detectable endogenous mRNA activity (data not shown). The amount of trichloroacetic acid-precipitable, radioactive procollagen formed was determined by bacterial collagenase digestion (Table 3). Analysis of translation products on gels showed production of full-size pro α chains from all RNA samples (not shown). Because each assay mixture contained an equivalent amount of total RNA, mainly rRNA (34), which was fairly constant in all lines, specific activity expressed as incorporation per μg of RNA was used to compare translatable mRNA levels in different cell samples.

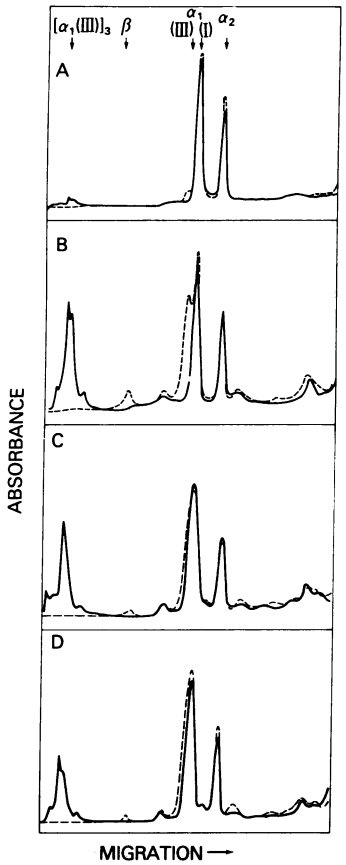

FIG. 3. Gel electrophoresis of collagen from control and tsKi-MSV-transformed cultures at nonpermissive and permissive temperatures. Cultures were grown as described in the legend to Fig. 2 and labeled for 3 hr at the same temperature used for preincubation, and collagen from the media was analyzed by gel electrophoresis with (– – –) and without (——) prior reduction by dithiothreitol. Scans of fluorograms are shown. (*A*) 3T3-714 grown at 36°C. (*B–D*) tsKi-3T3-714 grown at 36°C (*B*), 40°C for 24 hr (*C*), or 40°C for 48 hr (*D*).

While translation of RNA from transformed cells maintained at 36°C resulted in a lower relative rate of procollagen synthesis than was obtained with control cell RNA, this decrease was largely due to an increased level of mRNA for noncollagenous protein, rather than a proportionate decrease in procollagen mRNA. Transformed cells preincubated at 40°C contained increased procollagen and noncollagen protein mRNA activity reative to that present at the permissive temperature, but because these levels were similar to those of the untransformed cells at 40°C, the relative rates of procollagen synthesis directed by mRNA from control and transformed cells were almost identical.

Table 3. Procollagen synthesis from total cellular RNA in a reticulocyte lysate system

Source of RNA	Preincubation temperature, °C	Incorporation, cpm × $10^{-2}/\mu$g RNA: Collagen	Incorporation, cpm × $10^{-2}/\mu$g RNA: Noncollagen protein	Procollagen synthesis, %
(MLV)-3T3-P3	36	15.3	491	0.83
tsKi(MLV)-3T3-P3	36	10.7	826	0.35
(MLV)-3T3-P3	40	16.9	1149	0.39
tsKi(MLV)-3T3-P3	40	18.3	1364	0.36

Cells were either grown at 36°C or shifted to 40°C for 20 hr before total RNA was extracted.

DISCUSSION

The results described here rule out cell selection as a viable mechanism for the previously reported (5) difference in the collagen phenotype of MSV transformants. There was an increased proportion of type III, relative to type I, procollagen in two separate subclones of BALB 3T3 that were productively infected and transformed, conditions that eliminate selection of transformed clones. Infection with helper MLV alone neither transformed the cells nor altered the collagen phenotype, indicating a specific effect of Ki-MSV. An increased proportion of type III procollagen was also observed in a transformed nonproducer clone (tsKi-3T3-714) derived from the productively infected transformed culture of one of these subclones, 3T3-714, indicating that virus production is not required for this effect.

The low relative rate of procollagen synthesis characteristic of transformed 3T3 lines (5) also was observed in the productively infected/transformed cells and resulted directly from viral transformation. While the decreased relative rate of procollagen synthesis resulting from transformation of chicken embryo cells by RSV (6, 8) corresponded with a decreased level of procollagen mRNA (8), in the Ki-MSV-transformed cells it appears to result from a relatively small decrease in functional procollagen mRNA with a simultaneous large increase in mRNA active for noncollagen protein synthesis. The decreased relative rate of collagen synthesis was found to be temperature sensitive in cell culture and also by translating mRNA from cells grown at the two different temperatures. Thus, it appears that the alteration in the level of procollagen is regulated by the product of the *src* gene.

Surprisingly, the altered collagen phenotype did not revert to the nontransformed state even after 48 hr at the nonpermissive temperature, when cellular morphology, the ability to grow in soft agar, and the increased relative rate of procollagen synthesis had reverted. These results could mean that the altered collagen phenotype did not result from a *src* protein-initiated reaction or that fully functional *src* protein is not required to maintain the property because of a specifically mediated reaction that is either slowly reversible or irreversible, a change similar to terminal differentiation. Differences in the time required for temperature reversal of properties of tsRSV-transformed chicken embryo fibroblasts varies; glucose transport reverts in 6 hr (35), while procollagen and fibronectin mRNA levels return to normal by 24 hr (7). Alternatively, type III procollagen mRNA may be long-lived so that its translation continued at the nonpermissive temperature in spite of the absence of functional *src* protein. The half-life of total procollagen mRNA was reported to be 3 hr (36), but there are no such studies on individual procollagen mRNAs. There is also a possibility that the presence of Ki-MSV DNA is not required to maintain the altered collagen phenotype. We previously reported that a flat revertant of a Ki-3T3 line retained this property (33), and recent results revealed that the revertant has lost viral DNA (E. Scolnick, personal communication).

Apart from the question of whether *src* protein induces the alteration in the collagen phenotype, there are several possible ways in which this change could be achieved. We ruled out MSV induction of extracellular, but not intracellular, type I procollagen-specific degradation as an explanation. An alternative possibility would be that the synthesis of type I procollagen decreased after transformation while synthesis of type III continued at the same rate. The results of mRNA translation, however, revealed that the level of procollagen (mainly type I) mRNA in transformed cells was reduced by only 30%, so that even if the rate of type III synthesis remained the same as in nontransformed cells, its proportion would increase by only a few percent, not by the 4- to 10-fold observed. This leaves open the possibility that Ki-MSV transformation induces an increase in procollagen type III mRNA.

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