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Specific changes in the collagen phenotype of BALB 3T3 cells as a result of transformation by sarcoma viruses or a chemical carcinogen

(collagen types/4-nitroquinoline 1-oxide/simian virus 40)

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ABSTRACT **Radioactive proline-labeled procollagen, accumulated during a 3-hr incubation of normal and transformed BALB 3T3 cultures, was treated with pepsin and the resulting collagen components were analyzed by carboxymethyl-cellulose chromatography and sodium dodecyl sulfate/polyacrylamide gel electrophoresis in the presence or absence of reducing agent. Collagen in the medium of three subclones of BALB 3T3 A-31 that exhibited contact-inhibition of growth at confluence, as well as in the medium of one that did not, consisted of α_1 and α_2 subunits in the ratio of 3:1, suggesting that 3T3 cells synthesize type I collagen, $[\alpha_1(I)]_2\alpha_2$, and another type, which we have designated X, composed of α_1 chains, which may or may not be identical to $\alpha_1(I)$. Culture medium from 3T3 transformed by Kirsten or Moloney sarcoma virus contained type I collagen and another type differing from I and X and designated as type Y. The latter appeared to be similar to type III collagen $[\alpha_1(III)]_3$, since it contained intrahelical disulfide bonds. Analysis of intracellular collagen also demonstrated the presence of type III in Ki-3T3 and its absence from 3T3 cells. Collagen components from the medium of a simian virus 40 transformant were identical to those of the contact-inhibited clones, while the collagen from a 4-nitroquinoline-1-oxide-induced transformant was composed mainly of two components differing from $\alpha_1(I)$, α_2, or $\alpha_1(III)$. These results suggest that the type of collagen accumulated in transformed cell cultures may be specifically related to the transforming agent.**

Transformation of cells causes significant changes in cell morphology, growth rate, and synthesis of various cell components. These changes include decreased collagen (1–3) and acidic glycosaminoglycan (4, 5) synthesis, although hyaluronic acid synthesis often increases (6, 7). Recent work has revealed that there are at least five genetically specified collagen subunits (α-chains) which combine to form four different collagen molecules (8). Type I collagen, $[\alpha_1(I)]_2\alpha_2$, is ubiquitous; type II, $[\alpha_1(II)]_3$, is specific to cartilage; type III, $[\alpha_1(III)]_3$, is found in blood vessels, uterus, fetal skin, and other soft tissues, and is unique in having intrahelical disulfide bonds; type IV is contained in basement membrane and may have the composition $[\alpha_1(IV)]_3$ (9), although recent reports suggest heterogeneity (10, 11). It has been shown that there are changes in collagen types synthesized in pathological conditions (12). It was therefore of interest to determine whether transformation affects the type of collagen synthesized in addition to altering the rate of synthesis. In this study we determined the types, in addition to the amount, of collagen synthesized by the parent 3T3 lines and by transformants produced by sarcoma (RNA) and simian virus 40 (SV40) (DNA) viruses as well as by a chemical carcinogen, 4-nitroquinoline-1-oxide.

Abbreviations: CM-cellulose, carboxymethyl-cellulose; $NaDodSO_4$, sodium dodecyl sulfate; SV40, simian virus 40.

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MATERIALS AND METHODS

Cell Cultures. The cell lines used in this study were derived from BALB 3T3 A-31 and are shown in Table 1. BALB 3T3 tends to lose contact-inhibition (3); therefore subclones exhibiting contact-inhibition at confluence (P-1 and P-3) as well as a nondensity-dependent clone (P-13) were isolated in this laboratory. Cells in groups A and C formed a sheet with a cobblestone-like appearance at confluence while transformed cells did not, but 3T3-BB cells, a culture of nonsubcloned A-31 obtained from William Brockman, showed scattered areas of dense growth overlying the monolayer. The saturation density of clones in group A was approximately 6×10^4 cells per cm^2, while those in the other groups had saturation densities ranging from 17 to 20×10^4 cells per cm^2. Cells were cultured as previously described (3), except that gentamicin (50 μg/ml) replaced penicillin and streptomycin. Medium without serum is designated as MEM-0 and contained gentamicin but no Fungizone.

Incorporation of Radioactive Proline. Late logarithmic phase cells were used, except for the experiment in which collagen synthesis was examined at various stages of growth of 3T3 P-3. In this case, cells were used immediately after inoculation (lag phase) and in logarithmic and stationary phase (5th day of culture). Cells were labeled as described (3) in 3 ml of MEM-0 which contained 0.10 mM sodium ascorbate and 0.5 mM β-aminoproprionitrile, which inhibits formation of lysine-derived crosslinks between α-chains. After a 15-min preincubation at 37°, 7.5 μCi of [^{14}C]proline (50 μCi/μmol) or 75 μCi of [2,3-^{3}H]proline (500 μCi/μmol) was added to each dish, which was then incubated at 37° under 95% air/5% CO_2 for 3 hr. Cell and medium fractions were prepared as described (17). Lyophilized medium was dissolved in 0.1 M Tris·HCl buffer, pH 7.6, containing 0.4 M NaCl; 5–10% of the solution was saved for analysis with protease-free collagenase (17, 18), while the remainder was used to purify collagen for analysis on carboxymethyl(CM)-cellulose chromatography and sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis, as described below.

Purification of Radioactive Collagen. Lyophilized medium fraction was dissolved in 0.1 M Tris·HCl/0.4 M NaCl at pH 7.6, and collagen was precipitated by adding 176 mg/ml of ammonium sulfate. The precipitate was collected by centrifugation and dissolved in the Tris·HCl/NaCl solution. In some experiments, a portion of this solution was analyzed for procollagen by CM-cellulose chromatography and $NaDodSO_4$/polyacrylamide gel electrophoresis as described below. In most instances, the solution was dialyzed against 0.5 M acetic acid and then treated with 100 μg/ml of pepsin for 6 hr at 15°, which gave

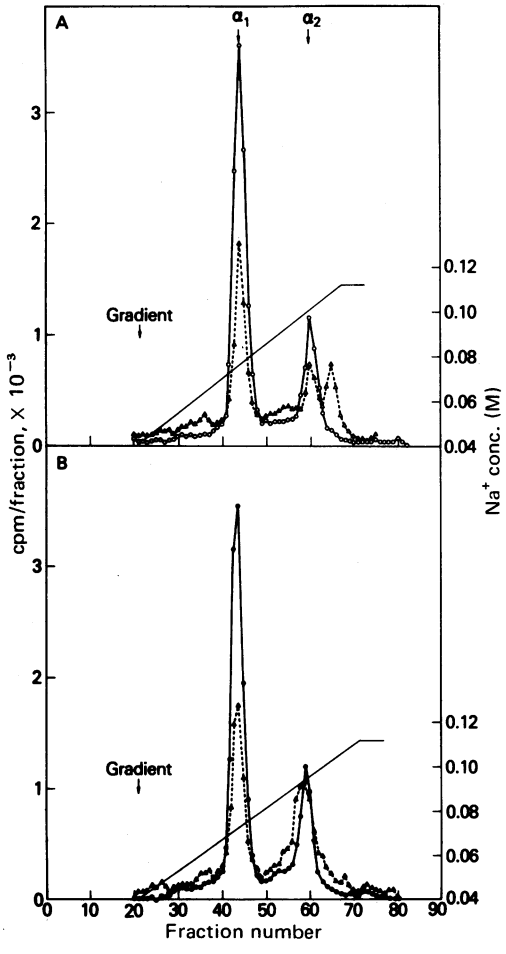

FIG. 1. CM-cellulose chromatograms of collagen from 3T3 and Ki-3T3 cells. Radioactive collagen from the medium of 3T3 P-1 (2×10^4 cpm of ^{14}C) and Ki-3T3-234-21 (1.8×10^4 cpm of ^3H) cultures was prepared as described under *Materials and Methods*, mixed, and applied to a CM-cellulose column (0.9 × 10 cm). The column was washed with starting buffer (0.04 M sodium acetate/4 M urea at pH 4.8), then developed with a NaCl concentration gradient from 0 to 72 mM with 50 ml each of starting and limit buffer at 43°. (*A*) Unreduced; (*B*) plus 1 mM dithiothreitol. (O—O, ●—●) 3T3 cells; (Δ - - Δ, ▲ - - ▲) Ki-3T3 cells. Arrows show the elution position of pepsin-treated BALB/c mouse skin collagen subunits. (——) Na^+ concentration.

maximum proteolysis of procollagen. Pepsin was inactivated by neutralization, the solution was dialyzed against 0.1 M Tris·HCl/0.4 M NaCl at pH 7.6, and collagen was reprecipitated with ammonium sulfate as above. Unlabeled collagen for reference was prepared from BALB/c weanling mouse skin by acetic acid extraction followed by NaCl precipitation (19). The collagen was treated with pepsin and subunit components were isolated by CM-cellulose chromatography.

CM-Cellulose Chromatography. CM-cellulose chromatography under denaturing conditions was used as described (20) except that 3.5 mg of calf skin acid-soluble collagen (Calbiochem) was used as carrier with each sample. Duplicate samples were incubated with or without protease-free collagenase before application to the column. Results are presented as the difference between the curves obtained with the two samples. Proportions of the major peaks were determined by planimetry.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Partially purified collagen fractions were dialyzed against 0.15 M acetic acid, lyophilized, and taken up in buffer composed of 0.04 M

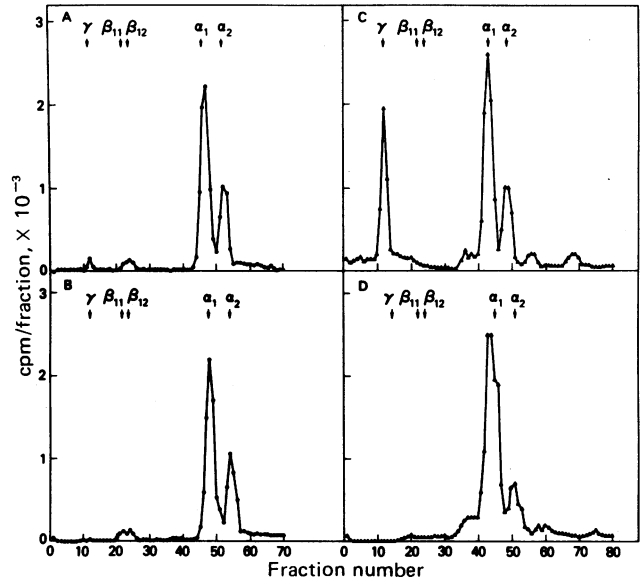

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of collagen from the media of 3T3 and Ki-3T3 cells. Radioactive collagen (1.4×10^4 cpm of ^{14}C and 2.1×10^4 cpm of ^3H) was prepared as described in the legend to Fig. 1 and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis as described under *Materials and Methods*. (*A*) [^{14}C]3T3, unreduced; (*B*) [^{14}C]3T3, with 1 mM dithiothreitol; (*C*) [^3H]Ki-3T3, unreduced; (*D*) [^3H]Ki-3T3, with 1 mM dithiothreitol. Arrows show the position of carrier collagen components from mouse skin. Migration is towards the right.

Tris-acetate (pH 6.3)/1% $NaDodSO_4$/1 mM EDTA; 25 μg of mouse skin collagen was added and the sample was heated at 65° for 30 min. Electrophoresis of samples was carried out using Bio-Phore precast 4% gels (Bio-Rad) with 0.21 M Tris-acetate/0.1% $NaDodSO_4$ at pH 6.3 for 30 min at 3 mA per gel and then for 4 hr at 6 mA per gel with or without dithiothreitol at 10 mM in the sample buffer and 1 mM in the electrophoresis buffer. Gels were fixed, stained and destained, and scanned at 550 nm; 1-mm gel slices were prepared as described (21). Recovery of radioactive collagen was determined using collagenase digestion, and was 90% before and about 80% after the staining and destaining procedure. Duplicate samples were analyzed either without or with collagenase pretreatment. Electropherograms display the difference between the two analyses. Proportions of the main peaks were determined as described above. Details of the above methods will be published elsewhere (R. Hata and B. Peterkofsky, unpublished).

RESULTS

Collagen Components Synthesized by BALB 3T3 Cells and Sarcoma Virus-Transformed Cells. The collagenous material secreted into the medium of BALB 3T3 (P-3) and Kirsten virus transformant Ki-3T3 (234-21) was present mainly as procollagen, as has been found for other cultured cells (22). Recovery of procollagen components from a CM-cellulose column was less than 50%, but pepsin treatment increased the recovery to about 90%; therefore, only pepsin-treated material was used for analyses described below. Fig. 1 shows the CM-cellulose chromatograms of collagen from [^{14}C]proline-labeled 3T3 and [^3H]proline-labeled Ki-3T3 culture media. The major collagen components from 3T3 medium corresponded to pepsin-treated α_1(I) and α_2 chains of mouse skin collagen, but the ratio of α_1:α_2 was 2.9 (Fig. 1*A*). In contrast, α_1 and α_2 components from Ki-3T3 medium were present in the ratio of 2.3 and there was an additional major component, designated Y, which eluted after

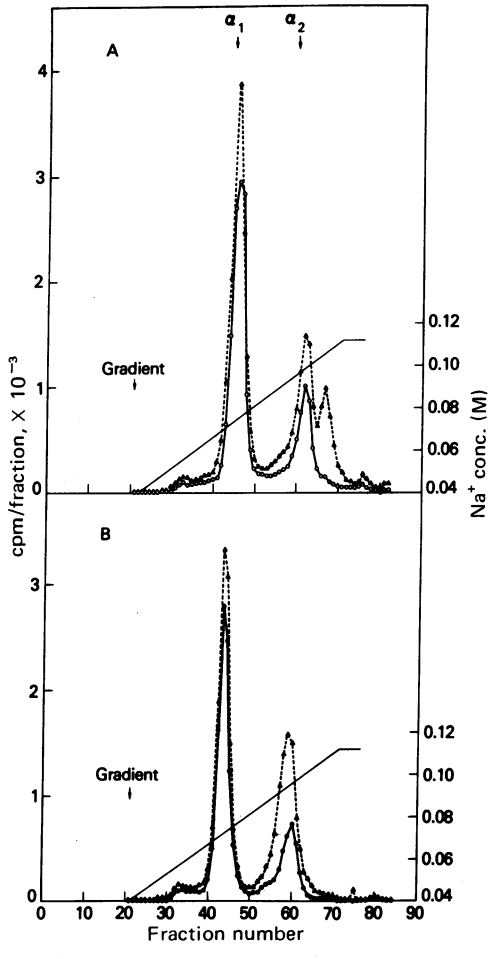

FIG. 3. CM-cellulose chromatogram of collagen from Mo-3T3 and 3T3 P-13. Radioactive collagen from the medium of 3T3 P-13 (1.8 × 10^4 cpm of ^{14}C) and Mo-3T3 cultures (3.0 × 10^4 cpm of ^3H) was mixed and analyzed on CM-cellulose as described in the legend to Fig. 1. (*A*) Unreduced: (O—O) [^{14}C]3T3 P-13; (Δ - - Δ) [^3H]Mo-3T3. (*B*) With 1 mM dithiothreitol: (●—●) [^{14}C]3T3 P-13; (▲ - - ▲) [^3H]Mo-3T3.

α_1 chains (Fig. 1*A*). Type I collagen labeled with radioactive proline should have an α_1:α_2 ratio of 2.3 since α_1(I) chains have a higher proline content than α_2 chains (8). In the presence of 1 mM dithiothreitol (Fig. 1*B*), the elution pattern of 3T3 collagen components was unchanged, but in the case of Ki-3T3 the elution position of the Y component changed, appearing slightly ahead of α_1. Two other subclones of 3T3 exhibiting contact-inhibition (P-3 and 714) and another isolate of Ki-3T3 (clone 1) were tested and gave almost identical results. The collagen components of BALB 3T3 and Ki-3T3 were further characterized by NaDodSO$_4$/polyacrylamide gel electrophoresis. By this analysis, it also was found that 3T3 contained α_1 and α_2 chains in the ratio of 2.6 and that the collagen from Ki-3T3 contained a component that moved as a trimer of collagen subunits or γ component (Fig. 2 *A* and *C*). In the presence of dithiothreitol, the mobilities of the components from 3T3 were unchanged (Fig. 2*B*), but the γ component of Ki-3T3 disappeared and the same amount of radioactivity appeared in the region of α_1 chains (Fig. 2*D*). These results indicated that the γ component, like the Y component, contained intrahelical disulfide bonds and, since each was the third major component in addition to α_1 and α_2, we concluded that γ and Y were identical.

Collagen in the medium accounted for 70% of the total radioactive collagen accumulated in 3 hr by 3T3 cells but for only 40% of Ki-3T3 collagen. Therefore, collagen from the cell fractions was also analyzed by CM-cellulose chromatography and NaDodSO$_4$/polyacrylamide gel electrophoresis. It was observed that type Y occurred in Ki-3T3 but not in 3T3 cells, while type I was present in both (data not shown).

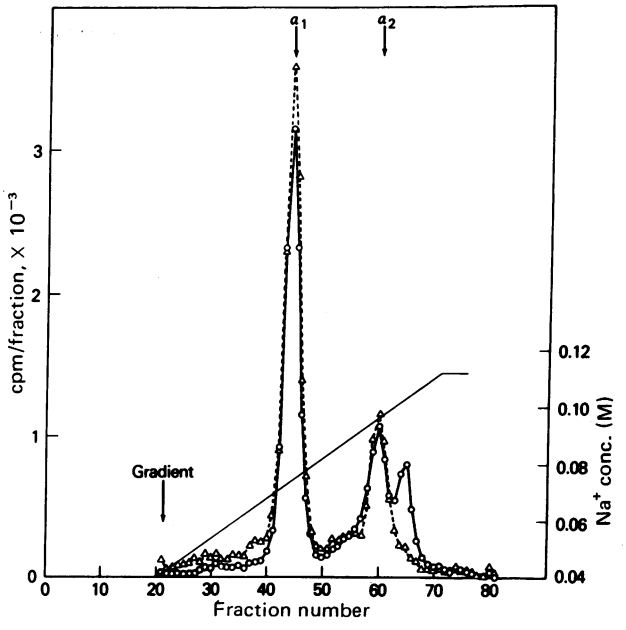

FIG. 4. CM-cellulose chromatogram of collagen from SV-3T3 and 3T3 A-31. Radioactive collagen from the medium of 3T3 A-31 (2.2 × 10^4 cpm of ^{14}C) and SV-3T3 (2.1 × 10^4 cpm of ^3H) cultures was mixed and analyzed on CM-cellulose as described in the legend to Fig. 1. (O—O) [^{14}C]3T3 A-31 (BB); (Δ - - Δ) [^3H]SV-3T3.

Specificity of Change in Collagen Phenotype. Experiments were carried out to determine whether the difference in the collagen types found in contact-inhibited compared to Kirsten sarcoma virus-transformed 3T3 cells was a phenomenon related specifically to viral transformation or generally to a rapid growth rate and loss of density-dependent growth control. Collagen components from 3T3 transformed with another sarcoma virus, Moloney (Mo-3T3), and from a nondensity-dependent subclone of 3T3 (P-13) were analyzed together on CM-cellulose. Collagen components from Mo-3T3 culture medium were similar to those of Ki-3T3 (Fig. 3). Although 3T3 P-13 cells have a faster growth rate and higher saturation density than clones 1, 3, and 714, the components of P-13 collagen were essentially the same as those of the three contact-inhibited clones. Results from NaDodSO$_4$/polyacrylamide gel electrophoresis supported this conclusion (data not shown).

Collagen from culture media of 3T3 cells transformed with SV40, a DNA virus, (SV-3T3) and from the 3T3-BB culture used for the transformation experiment were analyzed by CM-cellulose chromatography (Fig. 4). The chromatogram of the collagen from SV-3T3 was similar to that of the contact-inhibited subclones, but the chromatogram of collagen from 3T3-BB showed the presence of Y component in addition to α_1 and α_2 chains. Because of these unexpected results, this experiment was repeated using SV-3T3 from another passage, level and almost identical results were obtained. Component Y of 3T3-BB appeared to be identical to Y of Ki-3T3 since the peak moved to a position slightly ahead of the α_2 region when chromatography was carried out in the presence of dithiothreitol.

Collagen components from the medium of cells chemically

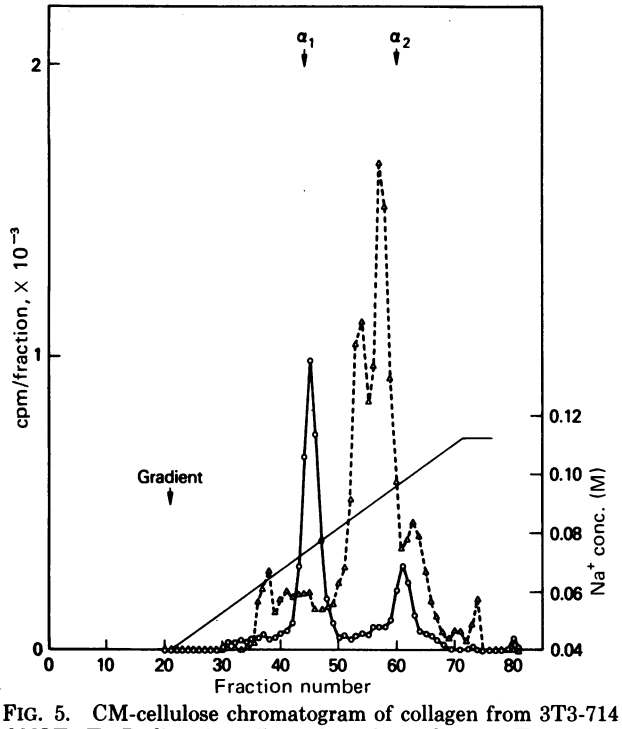

FIG. 5. CM-cellulose chromatogram of collagen from 3T3-714 and NQT-3T3. Radioactive collagen from the medium of 3T3-714 (6.1 × 10^3 cpm of ^{14}C) and chemically transformed NQT-3T3 (1.6 × 10^4 cpm of ^3H) cultures, which was repurified by ammonium sulfate precipitation after pepsin digestion, was mixed and analyzed on CM-cellulose as described in the legend to Fig. 1. (○—○) [^{14}C]-3T3-714; (△ - - △) [^3H]NQT-3T3.

transformed by 4-nitroquinoline-1-oxide (NQT-3T3) and from that of the contact-inhibited subclone 714 used as the parent in the transformation experiment were also compared by CM-cellulose chromatography (Fig. 5). The components of 3T3-714 were essentially the same as those of subclones 1 and 3, while NQT-3T3 contained essentially no α_1(I) or α_2 components and, instead, exhibited two main components which eluted between the α_1(I) and α_2 markers. In addition, there was a third peak slightly behind α_2, which may be type Y. These components were not characterized further, but the elution positions of the major components and their presence in the ratio of approximately 2:1 appears to be similar to collagen components recently isolated from fetal membrane (23) and skin basement membrane (11).

Table 1 lists the relative rates of collagen synthesis determined during these experiments for the various cell lines used. The contact-inhibited subclones, as well as the original A-31 clone and the nondensity-dependent subclone P-13, had rates between 0.9 and 2.0% while those of the transformants ranged from 0.45 to 0.74%, confirming previous observations that transformation reduces the relative rate of collagen synthesis (1–3).

DISCUSSION

We have shown that sarcoma virus-transformed or chemically transformed BALB 3T3 cell cultures contain collagen components different from those in cultures of BALB-3T3 subclones which exhibit strict contact-inhibition at confluence; the relative amounts of these components is summarized in Table 2. As discussed above, the expected α_1:α_2 ratio for proline-labeled type I collagen is 2.3. The contact-inhibited clones (P-1, P-3, and 714), regardless of growth stage, contained mainly type I collagen but the ratio of α_1:α_2 was close to 3, indicating an excess of α_1 chains. This suggests the presence of about 20% type X collagen, which could represent either cartilage collagen composed of three α_1(II) chains, since these elute at the same position as α_1(I) on CM-cellulose, or another type of collagen composed of three α_1 chains either identical or very similar to α_1(I). The latter type of molecule is synthesized by chondrocyte cultures treated with BrdUrd (24) and by fibroblasts from diseased human gingiva (25). Although difficult to determine from CM-cellulose chromatograms, a small amount of γ component was consistently observed by $NaDodSO_4$/polyacrylamide gel electrophoresis. In contrast, in sarcoma virus-transformed cells, Ki-3T3 and Mo-3T3, type Y collagen appeared to replace type X as a major component. The behavior of type Y collagen in $NaDodSO_4$/polyacrylamide gel electrophoresis indicated that, like type III collagen, it is a trimer of α_1 components held together by intrahelical disulfide bonds. In addition, its elution on CM-cellulose chromatography is very similar to that of pepsin-treated, rat skin type III collagen (26), but further chemical analysis will be required to confirm the tentative conclusion that Y is type III collagen.

Table 1. BALB 3T3 cell lines used

	Abbreviation	Type of cell	Doubling time, hr	Relative rate of collagen synthesis, %*	Source and ref.
(A)	3T3 P-1	Contact-inhibited subclone of A-31	16	1.90	†
	3T3 P-3	Contact-inhibited subclone of A-31	20	1.96	†
	3T3 714	Contact-inhibited subclone of A-31	22	0.91	(13)
(B)	3T3 P-13	Nondensity-dependent subclone of A-31	14	1.10	
(C)	3T3 BB	Original 3T3 A-31, not subcloned	20	1.47	(14)
(D)	Ki-3T3-1	Kirsten virus-transformed	16	0.56	(15)
	Ki-3T3-234-21	Kirsten virus-transformed	14	0.41	(15)
	Mo-3T3	Moloney virus-transformed	19	0.74	(16)
(E)	SV-3T3	SV40-transformed (WTB3b)	13	0.61	‡
(F)	NQT-3T3	4-Nitroquinoline-1-oxide-transformed (NQT-1)	12	0.45	(13)

* Calculated assuming that collagen has an imino acid content 5.4 times higher than other proteins (18) and is the percent radioactive proline incorporated into collagen compared to total protein in 3 hr.
† B. Peterkofsky and W. Prather, unpublished results.
‡ W. Brockman, unpublished results.

Table 2. Proportions of major collagen components in the medium of BALB 3T3 and derivative cell lines*

Cell line	No. of analyses†	α_1	α_2	Y	α_1:α_2
3T3 P-1	2	70.3	25.2	4.5	2.8
3T3 P-3					
Lag phase	1	74.2	23.6	2.2	3.1
Log phase	2	70.8	26.1	3.1	2.7
Stationary phase	1	70.6	25.5	3.9	2.8
3T3-714	1	73.4	23.0	3.6	3.2
3T3 P-13	2	69.4	27.0	3.6	2.6
SV-3T3	2	69.7	24.4	5.9	2.9
3T3-BB	2	58.6	23.7	17.7	2.5
Ki-3T3-1	2	48.4	21.3	30.3	2.3
Ki-3T3-234-21	5	52.4	23.3	24.3	2.3
Mo-3T3	2	59.5	26.3	14.2	2.3

* Results for NQT-3T3 were not included since the major components did not correspond to α_1 and α_2 chains.

† In cases where more than one analysis was carried out, results represent average values and include both CM-cellulose chromatography and NaDodSO$_4$/polyacrylamide gel electrophoresis.

The difference in collagen components described above apparently is not related to the faster growth rate or higher saturation densities of the RNA virus or chemically transformed cells, since 3T3 P-13 cells, which have a shorter doubling time and higher saturation density than clones 1, 3, and 714, produced types of collagen similar to those of the contact-inhibited cells. The change from type X to type Y collagen may be specific for sarcoma virus transformation, since an SV40 transformant contained the same types of collagen as 3T3 and the main types of collagen components accumulated by a chemical carcinogen-induced transformant (NQT-3T3) were quite different from those of the parent 3T3-714 as well as from those of the sarcoma virus transformants, although type Y may also be present. This specificity is unusual since the characteristic transformed phenotype generally is acquired without regard to the transforming agent (27). There is previous experimental evidence, however, that supports the concept that transformation by DNA and RNA viruses may proceed via different pathways. Renger (28) found that an SV40-transformed, temperature-sensitive 3T3, with the defect in the host rather than the virus, exhibited the transformed phenotype at 32° but not at 39° and could be retransformed at 39° by Moloney sarcoma virus but not by Sv40. Recently, Todaro *et al.* (29) reported that activity of epidermal growth factor receptor was lost from various cell lines, including BALB 3T3 A-31, after transformation by murine and feline sarcoma viruses but not by SV40. This specificity is very similar to that which we observed for the change from type X to type Y collagen. There are at least two ways in which this change in collagen types might be explained: (*i*) infection by sarcoma viruses or insertion of the sarcoma virus genome results in activation of host genes involved in synthesis or regulation of collagen or (*ii*) variant cells within the heterogeneous 3T3 A-31 population may synthesize type Y collagen and be selected for by sarcoma virus transformation. This implies that cell types not synthesizing type Y may not be susceptible to transformation by sarcoma virus but may be susceptible to SV40 transformation. The finding that 3T3 A-31, which had not been subcloned, contained approximately 18% type Y collagen while subclones 1, 3, and 714 contained at most 2–4% gives some support to this possibility, although spontaneous transformation of the culture between the time of the original SV40 transformation experiment and analysis for collagen components might explain this result. Analysis of the collagen from additional subclones of 3T3 A-31 and from transformants produced by other viruses and carcinogens should establish whether the specificity observed here is general and whether the effect of transformation with sarcoma virus is due to integration of viral DNA or selection.

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