Epithelial-like chondrocytes obtained from chick embryo were transformed with Rous sarcoma virus. Cellular transformation was monitored looking at the morphology change, the cell growth, and the expression of plasminogen activator.

Analysis on polyacrylamide gel of intracellular and secreted proteins showed: 1) a disappearance of the specific products of differentiated chondrocytes; 2) a switch in the collagen synthesis from type II, the chondrocyte-specific type, to the type I, characteristic of fibroblasts and other cells of mesenchymal origin; 3) an enhancement of fibronectin synthesis.

Analysis of the proteins from chondrocytes infected with Rous-associated virus I, a virus unable to induce cell transformation in vitro, indicated that the altered expression of the differentiated proteins in Rous sarcoma virus-infected chondrocytes depended upon the action of src gene product.

EXPERIMENTAL PROCEDURES

Cell Cultures and Viral Infection—The preparation of chondrocyte cultures from tibiae of 19-day-old White Leghorn chick embryos (SPF eggs, Lohmann Toerzucht GMBH, Cuxhaven, Germany) as well as the preparation of media and the source of materials have been previously described (1). Fibroblasts were prepared according to Pfeifferkorn and Hunter (13) and grown in F 190 medium supplemented with 4% calf serum, 1% chicken serum, and 10% tryptose phosphate broth (Difco).

Epithelial-like cells grown in the absence of ascorbic acid (5 × 10⁴ cells per 35 mm dish) were infected in monolayer 24 h after plating with a small volume of virus suspension at a multiplicity of infection of approximately 20 (20 m.o.i.). After 1 h the cultures were given 2 ml of fresh medium; medium was changed after 1, 4, 8, and 12 days. Alternatively the epithelial-like cells were harvested with a mixture of 0.25% trypsin and 100 units/ml of collagenase II and infected with the virus at 2 m.o.i. in a small volume for 1 h; infected and mock infected chondrocytes were replated and fresh medium was added. Fibroblasts were always infected in suspension at 2 or 20 m.o.i. A clonal isolate from the SR-RSV of subgroup A served as nd virus; RAV-1 served as lymphoid leukemia virus (14). Both viruses were obtained from Dr. Philippe Vigier, Fondation Curie, Orsay, France.

Infected Cell Assay—The cells were washed twice with phosphate-buffered saline, detached from the dishes with the trypsin-collagenase mixture, and counted. Serial dilutions of the cells were plated on monolayers of chick embryo fibroblasts (3 × 10⁴ cells per 35-mm dish) and after 1 h the fibroblasts were overlaid with medium containing agar at 0.75% final concentration. Dishes were incubated at 37°C. Fibroblasts cultures were scored for transformation foci under the microscope after 7 days.

Fibrinolysis Assay—Fibrinolytic activity was assayed according to Wigler and Weinstein (15) using 10⁴-fibrin-coated plastic Linbro multiwells. The assay was performed on cell lysates in phosphate-buffered saline containing 0.1% Triton X-100 and on culture media
harvested 24 h after the cells were fed. To inactivate acid-sensitive protease inhibitors, the media were acidified with 1 N HCl and neutralized with 1 N NaOH before the assay (16). We assayed each sample in triplicate at four time points and considered for the final result only the points in the time interval at which linearity was observed. One unit of activity was defined as the amount of enzyme catalyzing the release of 1 mg of fibrin at 37 °C in 1 h.

**Collagen and Fibronectin in Transformed Chondrocytes**

**RESULTS**

**Transformation of Chondrocytes**—Epithelial-like chondrocytes obtained from chick embryo were infected in suspension with SR-RSV A after digestion of the extracellular matrix. Infected cells presented the characteristically bipolar morphology of the transformed chondrocytes (Fig. 1). (8, 9). The infection and the release of virus was monitored with the infected cell assay. In Table I are reported the results of a typical experiment in which the percentages of infected cells in two parallel cultures of chondrocytes and fibroblasts were determined at different times. Consistent with their longer doubling time the spreading of infection was slower in chondrocytes. The number of the chondrocytes with bipolar morphology was proportional to the number of the virus-producing cells. When, after 14 days, 100% of chondrocytes released infectious virus, all cells presented a bipolar morphology. In some experiments epithelial-like chondrocytes were infected with a 10-fold higher viral concentration directly as monolayer, 24 h after plating, without previous digestion with proteolytic enzymes; we did not observe any major difference in the efficiency of transformation when the cultures were infected with the two procedures.

Transformation by RSV stimulated cell growth; in one experiment the doubling times of normal and infected chondrocytes were 2.8 and 1.5 days, respectively. In addition the final level of cell density in cultures of transformed chondrocytes was always at least 3 times higher than in cultures of mock infected cells. Transformed chondrocytes secreted plasminogen activator to an extent comparable to the one observed in transformed fibroblasts (Table II). We did not check the growth in soft agar of transformed cells since normal chondrocytes have already a relatively high colony-forming efficiency (data not shown).

**Protein Synthesis of Transformed Chondrocytes**—We have recently analyzed and partially characterized by collagenase digestion, immunoprecipitation, and pulse-chase experiments some of the proteins synthesized in large amount by cultured chondrocytes (1). Arrows in Fig. 2 refer to four of the proteins that we consider as specific differentiation markers; from top to bottom: 1) a high molecular weight protein, not recognized by antibodies raised against type I collagen, type II collagen, and fibronectin; 2) the pro-α1 (II) collagen; 3) a ~75,000 protein comigrating with chondronectin (18, 19) specifically immunoprecipitated from media; 4) a 64,000 collagenase-sensitive polypeptide.

[^35]S]Methionine-labeled intracellular proteins from normal and transformed chondrocytes were compared with proteins from normal and transformed fibroblasts (Fig. 2). Viral transformation resulted in the disappearance of the above recalled differentiation markers. In some experiments (see, for example, lane 3 of Fig. 5d) the disappearance of pro-α1(II) chain, induced by RSV transformation, is accompanied by the appearance of a polypeptide clearly detectable in the collagen region of the gel; since it comigrated with the major collagenase-sensitive polypeptide synthesized by cultured fibroblasts (arrowhead in Fig. 2) it was tentatively identified as pro-α1(I) collagen.

In **vivo** chondrocytes do not synthesize fibronectin (20, 21). In **vitro** the enzymatic dissociation of the extracellular matrix favors the synthesis of this protein (1, 20). The amount of fibronectin made by cultured chondrocytes can vary from undetectable to considerable levels. When chondrocytes that did not synthesize fibronectin were infected, the viral transformation resulted in the appearance on the gel of the 220,000 fibronectin band (Fig. 3, lanes 3 and 4 and Fig. 5d, lanes 1

[^2]: O. Capasso, unpublished data.
Collagen and Fibronectin in Transformed Chondrocytes

Table II

| Induction of plasminogen activator by Rous sarcoma virus in chick embryo chondrocytes and fibroblasts |
|-------------------------------------------------|
| Epithelial-like chondrocytes were infected as monolayer at 20 m.o.i. and assayed for plasminogen activator 14 days after infection. Fibroblasts were infected in suspension at 20 m.o.i. and assayed 4 days after infection. The fibrinolysis assay is described under “Experimental Procedures.” The amount of proteins in each lysate was determined and the weight of cells was deduced on the assumption that proteins represent 10% of the total cellular mass. |  |
| | Units of activity in 1 g of cells | Units of activity secreted in the medium in 24 h by 1 g of cells |
| Mock infected chondrocytes | 0.54 | 0.43 |
| Infected chondrocytes | 0.98 | 6.71 |
| Mock infected fibroblasts | 0.01 | <0.01 |
| Infected fibroblasts | 2.27 | 8.62 |

![Figure 2](http://www.jbc.org/)

**Figure 2. Intracellular proteins from normal and RSV-transformed chondrocytes and fibroblasts.** 1, normal chondrocytes; 2, RSV-transformed chondrocytes; 3, normal fibroblasts; 4, RSV-transformed fibroblasts. Cells were labeled when massively transformed, i.e., 14 days after infection for the chondrocytes and 5 days for the fibroblasts. Middle arrow refers to the pro-$\alpha$1(II) collagen. Arrows refer to the specific proteins of the differentiated chondrocytes (see text). Arrowhead refers to the pro-$\alpha$1(I) collagen. The numbers on the right refer to kilodaltons of molecular weight markers. The polyacrylamide concentration of the gel was 9%. All samples were reduced and alkylated before the electrophoresis.

![Figure 3](http://www.jbc.org/)

**Figure 3. Immunoprecipitation of intracellular proteins from normal and RSV-transformed chondrocytes and fibroblasts.** 1, 5, 9, proteins from normal fibroblasts. 2, 6, 10, proteins from RSV-transformed fibroblasts; 3, 7, 11, 13, proteins from normal chondrocytes; 4, 8, 12, 14, proteins from RSV-transformed chondrocytes. The immunoprecipitation was performed with antibodies from rabbit immunized with chicken fibronectin (Fn. Ab.), chicken type I collagen ($\alpha$1 Ab.), and chicken type II collagen ($\alpha$IIAb.). The lower molecular weight protein in lane 8 recognized by these antibodies is most probably a specific degradation product of the fibronectin, due to proteolytic activity present in the cell lysates. The polyacrylamide concentration of the gel was 7.5%. All samples were reduced and alkylated before the electrophoresis.

Collagen Secretion by Transformed Chondrocytes—The electrophoretic pattern of proteins secreted by control fibroblasts (Fig. 4, lane 3) revealed the presence of several polypeptides migrating in the collagen region of the gel. To identify these polypeptides the secreted proteins were immunoprecipitated with type I collagen antibodies (not shown) and the relative electrophoretic migrations of the immunoprecipitated polypeptides (i.e., all type I-related collagen chains named in the figure) were compared to the reported migrations of the type I procollagen and pC-collagen chains (26, 27). In addition to type I collagen, cultured chicken fibroblasts synthesize and secrete a relatively large amount of type III collagen (28). The pro-$\alpha$1(III) chain has an electrophoretic migration intermediate between the migrations of the pro-$\alpha$1(I) and pC-$\alpha$1(I) chains (29). Purified type I collagen antibodies do not cross-react with type III collagen (30); the band, not recognized by type I antibodies, was, therefore, tentatively identified as pro-$\alpha$1(III) collagen (full dot in Fig. 4).

When we compared the patterns of proteins secreted by normal and transformed chondrocytes (Fig. 4, lanes 1 and 2) to that of control fibroblasts, we observed that transformation of chondrocytes resulted in a dramatic decrease of the pro-$\alpha$1(II) chain and in the appearance of the pro-$\alpha$1(I) chain and of its maturation product pC-$\alpha$1(I). In overexposed autoradiographs, in the lane of proteins secreted by transformed chondrocytes we also observed polypeptides with the same electrophoretic migration of the pro-$\alpha$2(II) and pC-$\alpha$2(II) chains; however, the amount of radioactivity in these polypeptides was too small to allow any further analysis.

Infection of Chondrocytes with RA V-1—We performed some...
experiments with two temperature-sensitive transformation mutants of Rous sarcoma virus (ts-T PA3 and ts-T PA6 of Reference 31). At the nonpermissive temperature the large majority of the infected cells did not present the characteristic morphology of the transformed chondrocytes. Moreover we could oscillate between the normal and transformed phenotypes by simply shifting the incubation temperatures of the infected cells. In this condition the morphological reversion was obtained within 2–3 days. Nevertheless, due to the lea



FIG. 4. Secreted protein form normal and RSV-transformed chondrocytes and fibroblasts. 1, normal chondrocytes; 2, RSV-transformed chondrocytes; 3, normal fibroblasts. Transformed chondrocytes were labeled 14 days after infection. Arrows refer to specific proteins of the differentiated chondrocytes. The protein marked with full dot was tentatively identified as pro-α(1)(III) collagen (see text). The polyacrylamide concentration of the gel was 9%. All samples were reduced and alkylated before the electrophoresis. Since the same amount of trichloroacetic acid-precipitable counts was layered on each slot, each sample represented labeled proteins released in the medium by a different number of cells.

FIG. 5. RAV-1-infected chondrocytes. a, epithelial-like chondrocytes infected in suspension with RAV-1 at approximately 2 m.o.i. The picture was taken 10 days after infection × 256. b, RAV-1-infected chondrocytes passaged and superinfected after 7 days with SR-RSV A. The picture was taken 7 days after SR-RSV A superinfection × 256. c, control chondrocytes 7 days after infection with SR-RSV A × 256. d, intracellular proteins of 1, normal; 2, RAV-1-infected chondrocytes; 3, SR-RSV A-transformed chondrocytes. Cells were labeled 14 days after infection. The polyacrylamide concentration of the gel was 9%. All samples were reduced and alkylated before the electrophoresis.

FIG. 5d).

DISCUSSION

In this paper we report that in RSV transformed chondrocytes the synthesis of specific proteins of the differentiated status is repressed; in particular we observed the shut-off of type II collagen and of a 64,000 collagenase-sensitive protein we have recently identified (1). The shut-off of these proteins was accompanied by a switch on of the synthesis and secretion of type I collagen (namely of the α1(I) chain). The identification of the type Iα1 chain was based on its electrophoretic comigration with the collagen synthesized by cultured chicken fibroblasts and on its specific immunoprecipitation. Although in overexposed gels of proteins secreted by transformed chondrocytes one can observe polypeptides with the same electrophoretic mobilities of the α2(I) chains, we were unable to distinguish whether RSV transformation induces in the chondrocytes the only synthesis of α1(I) chain or whether the α1(I) chain is expressed preferentially in comparison to the α2(I) chain. We favor the second hypothesis; in fact, making use of a DNA probe (kindly provided by Drs. B. de Crombrugghe and I. Pastan, National Institutes of Health, Bethesda, MD) we obtained by dot blot hybridization preliminary data showing the appearance of the mRNA for the α2(I) chain in transformed chondrocytes.

Yoshimura et al. (11) have reported that transformation of chondrocytes resulted in a decreased amount of type II collagen synthesized but they failed to demonstrate any type I collagen chain in the proteins associated to the cells and secreted in the media. The carboxymethylcellulose chromatography they used to demonstrate the absence of α2(I) chains in the medium did not clearly separate α1(I) and α1(II) chains; a secretion of type I collagen α1 chain accompanied by a decrease in the synthesis of type II collagen could, therefore, have gone undetected. Adams et al. (12) have also found a reduction in the synthesis of type II collagen and, although they were able to show the presence of type I collagen mRNAs, they stated they were not able to detect any type I collagen...
synthesis in transformed chondrocytes. The authors did not report any attempt to reveal the presence of type I collagen in the lysates making use of specific antibodies or to analyze the proteins secreted in the medium.

In vivo chondrocytes do not express fibronectin. Cultured chondrocytes, after removal of the extracellular matrix, synthesize and secrete this protein in an extremely variable amount. We found that, after RSV transformation, chondrocytes always synthesized and released in the medium large quantities of fibronectin. The enhancement of the synthesis of fibronectin that we observed is in good agreement with the results obtained by the other groups (11, 12).

The switch between the collagen types and the enhancement of the fibronectin synthesis depended upon the action of src gene product; when we infected the cells with the lymphoid leukosis virus RAV-1 we failed to observe both phenomena. It must be noted that the effect of src gene in chicken chondrocytes is completely different from the effect of the same gene in chicken fibroblasts. In the latter cells the synthesis of fibronectin and type I collagen (and their mRNAs) is in fact strongly inhibited (Figs. 2 and 3; Refs. 22–25).

The block of the synthesis of specific products and the production of type I collagen and fibronectin in transformed chondrocytes could be interpreted as a consequence of a dedifferentiation process. On the other hand, it must be recalled that in developing limb buds an increase of fibronectin and type I collagen was detected by immunofluorescence in the cartilage blastema before the deposition of the extracellular matrix. These proteins disappeared in the mature cartilage when the presence of type II collagen was detectable (34). Given that, it is tempting to speculate that, as a consequence of the expression of src gene, cultured chondrocytes revert to a predifferentiated state.

In preliminary experiments, by immunofluorescence with antibodies against fibronectin, we observed a well developed extracellular network of fibronectin fibers in transformed chondrocytes. These data suggest that transformed chondrocytes incorporate fibronectin into their extracellular matrix, at variance with RSV-transformed fibroblasts (35). Understanding the reasons for this difference would greatly help to clarify the regulation of the synthesis and the role of the extracellular matrix in different types of cells.

Acknowledgments—We thank Gianfranco Pontarelli for technical assistance and Drs. Guido Tarone and Manfred Wiestner for the specific antibodies.

REFERENCES
1. Capasso, O., Gionti, E., Pontarelli, G., Amblesi-Impiomato, F. S., Nitsch, L., Tajana, G., and Cancetta, R. (1982) Exp. Cell Res. 142, 197–206
2. Boettiger, D., Roby, K., Brumbaugh, J., Biehl, J., and Holtzer, H. (1977) Cell 11, 881–890
3. Easton, T. G., and Reich, E. (1972) J. Biol. Chem. 247, 6420–6431
4. Fiersman, M. Y., and Fuchs, P. (1975) Nature (Lond.) 254, 429–431
5. Ephrussi, B., and Temin, H. M. (1960) Virology 11, 547–552
6. Fusco, A., Pinto, A., Tramontano, D., Tajana, G., Vecchio, G., and Tsachida, N. (1982) Cancer Res. 42, 618–626
7. Caloathy, G., Poirier, F., Dambrine, G., Mignatti, P., Combes, P., and Pessac, B. (1979) Cold Spring Harbor Symp. Quant. Biol. 44, 883–900
8. Okayama, M., Yoshimura, M., Muto, M., Chi, J., Roth, S., and Kaji, A. (1977) Cancer Res. 37, 712–717
9. Pacifici, M., Boettiger, D., Roby, K., and Holtzer, H. (1977) Cell 11, 891–899
10. Muto, M., Yoshimura, M., Okayama, M., and Kaji, A. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 4173–4177
11. Yoshimura, M., Jinninse, S. A., and Kaji, A. (1981) J. Biol. Chem. 256, 9111–9117
12. Adams, S. L., Boettiger, D., Focht, R. J., Holtzer, H., and Pacifici, M. (1982) Cell 30, 373–384
13. Pfeifferkorn, E. R., and Hunter, H. S. (1963) Virology 20, 433–445
14. Rubin, H., and Vogt, P. K. (1962) Virology 17, 184–194
15. Wigler, M., and Weinstein, I. B. (1976) Nature (Lond.) 259, 232–233
16. Luskinautoff, D. J., and Edgington, T. S. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3903–3907
17. Wiestner, M., Fischer, S., Dessau, W., and Muller, P. K. (1981) Exp. Cell Res. 133, 115–125
18. Hewitt, A. T. K., Kleinman, H. K., Pennypacker, J. P., and Martin, G. R. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 385–388
19. Hewitt, A. T. K., Varner, H. H., Silver, M. H., Dessau, W., Wilkes, C. M., and Martin, G. R. (1982) J. Biol. Chem. 257, 2330–2334
20. Dessau, W., Sasse, J., Timpl, R., Jilek, F., and von der Mark, K. (1978) J. Cell Biol. 79, 342–355
21. Lewis, C. A., Pratt, R. M., Pennypacker, J. P., and Hassel, J. R. (1978) Dev. Biol. 64, 31–47
22. Levinson, W., Bhatnagar, R. S., and Liu, T. Z. (1975) J. Nat. Cancer Inst. 55, 807–810
23. Olden, K., and Yamada, K. (1977) Cell 11, 957–969
24. Adams, S. L., Sobel, M. E., Howard, B. H., Olden, K., Yang, K. M., De Crombrugge, B., and Pestan, T. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3399–3403
25. Sandmeyer, S., Gallis, B., and Bornstein, P. (1981) J. Biol. Chem. 256, 5022–5028
26. Leung, M. K. F., Fessler, L. I., Greenberg, D. B. and Fessler, J. H. (1979) J. Biol. Chem. 254, 224–232
27. Prokop, D. J., and Tuderment, L. (1982) Methods Enzymol 82, 365–319
28. Hermann, H., Dessau, W., Fessler, L. I., and von der Mark, K. (1980) Eur. J. Biochem. 105, 63–74
29. Fessler, L. I., Timpl, R., and Fessler, J. H. (1981) J. Biol. Chem. 256, 2531–2537
30. von der Mark, H., von der Mark, K., and Gay, S. (1976) Dev. Biol. 48, 237–249
31. Giordi, E., Krycvse-Martine, C., Aupox, M. C., and Calothey, G. (1980) Virology 100, 219–228
32. John, R. H., Bielitter, M. A., and Weissmann, C. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 4772–4776
33. Tooze, J. (1973) in The Molecular Biology of Tumor Viruses (Tooze, J., ed) pp. 534–536, Cold Spring Harbor Laborator. Cold Spring Harbor, New York
34. Von der Mark, K. (1980) Curr. Top. Dev. Biol. 14, 199–225
35. Wartiovaara, J., Linder, E., Ruooslahti, E., and Vahehi, A. (1974) J. Exp. Med. 140, 1522–1533

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J. Biol. Chem. 1983, 258:7190-7194.

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