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Growth Characteristics of a Hamster Cell Line on Collagen Gel[1] (38095)

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(Introduced by W. H. Fishman)

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We have reported recently a hamster fibro-hemangiosarcoma whose tumor mass was dependent upon the sex of the host (1). Cells of this tumor have been placed in culture. This cell line is presently in its 59th passage and a full description will be reported when 70 passages have been completed and it may, for purposes of definition, be considered to constitute an established cell line. Animal inoculation of cells from this culture line have produced consistently a characteristic tumor believed to be of mesenchymal origin (1). Since collagenolytic activity by sarcoma tissues in culture has been reported (2) it seemed worthwhile to examine this cell culture for similar activity by observing its growth on firm surfaces of radioisotopically labeled reconstituted collagen gels (3). It was found however that, although the 2-31 line invaded the collagen matrix and produced clearing, there was no collagenolytic activity. Furthermore, continued growth of cells from this culture on collagen gels for periods up to four months produced condensation of the gel, a result not heretofore reported by previous investigators who, however, used other mammalian cell lines and shorter growth periods (4, 5).

Methods and Materials. Collagen gels. Collagen was labeled by injecting 3-wk-old white male rats with a total of 250 μCi of [^{14}C]glycine (U) (New England Nuclear, Boston, Mass.) over a period of 3 wk with the last injection of 25 μCi given 24 hr before sacrifice. Tendons were stripped from the tail and collagen extracted using 0.2 *M* acetic acid solution followed by a salt extraction with 0.1 *M* NaCl according to the method of Berman, Manabe, and Davison (6).

Following centrifugation, the soluble collagen in the supernatant was precipitated with 50% ammonium sulfate. The supernatant was discarded and the precipitated collagen solubilized with an appropriate volume of 0.05 *M* acetic acid to give a final concentration of 1–2 mg/ml. After extensive dialysis against 0.05 *M* acetic acid the pH was adjusted to 7.5–8.0 with 2 *M* Tris. Sufficient sodium chloride was added to make the solution 0.4 *M*. The final concentration of collagen was 0.125% as estimated using the Lowry method for protein determination (7). This fraction of soluble collagen had a specific activity of about 3200 cpm/mg protein.

Aliquots of approximately 2 ml of this collagen solution were distributed to 60 × 15 mm Falcon plastic culture dishes and heat gelation was carried out at 37°C. Sterilization of the gel was accomplished by exposure to an ultraviolet source at a dose level of about 20 ergs/mm^2/sec at a distance of 10 in. for 2 hr. Equilibration of the gels was carried out with a combination of Ham's F10 and F12 basal medium (1) at 37°C for 24 hr.

Cell cultures. Cells used in this study were from a culture derived from a hamster fibro-hemangiosarcoma previously reported (1).

"L" cells and HeLa cells were obtained from Microbiological Associates, Bethesda,

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Maryland, and grown on collagen gels in basal growth medium supplemented with 10% horse serum and 5% fetal calf serum, respectively.

Subcultures. Single cell suspensions were made from stock monolayer cultures by using 0.01% trypsin in phosphate buffered saline (PBS). Appropriate dilutions for plating were made with the basal medium supplemented with 10% horse serum. Each gel was seeded with about 5×10^4 cells and this cell number was estimated using a hemocytometer counting chamber according to the method of Sanford *et al.* (8).

Culture conditions. Growth medium was prepared from the best grade of commercially available chemicals, sterilized by Millipore filtration and stored at −10°C. The basal medium was a combination of Ham's F10 and F12 medium (1) and was supplemented with 10% horse serum procured from Grand Island Biological Co., Grand Island, N.Y. The growth medium was replaced every 84 hr with 3 ml of fresh growth medium. Incubation was at 37°C in a water-jacketed incubator with a water-saturated atmosphere of 95% air and 5% CO_2.

Detachment of cells. Cells grown upon firm gel surfaces of reconstituted ^{14}C-prelabeled collagen were removed by successive treatments of 10^{-3} *M* EDTA and 0.1% trypsin in PBS. This treatment was performed only after the collagen gel had lost its opalescence due to extensive cellular growth but prior to the complete detachment of the gel from the culture dish surface. Examination by phase microscopy showed that all surface cells had been removed but some cells had invaded the gel matrix and had remained intact. However, this represented a very small percentage of an average of 4×10^6 cells removed per culture dish. The removed cells were pelleted by centrifugation and the supernatant separated. The cells were washed repeatedly by alternating resuspension of the pellet in 3 ml of fresh PBS with subsequent centrifugation and separation of the supernatant. This process was continued until the radioactivity of the supernatant was reduced to the background level of 28 cpm. One milliliter of the supernatant was saved at each washing and was added to a scintillation vial with 19 ml of Aquasol and counted.

Assays. Collagenolysis. Collagenolysis by mammalian cells was determined by the release of soluble radioisotopically labeled collagen peptides into the growth medium (3) from ^{14}C-prelabeled reconstituted collagen gels prepared as described above. At each change of growth medium a 1 ml aliquot of the spent medium was placed in a scintillation vial with 19 ml of Aquasol (New England Nuclear, Boston, Mass.) and counted in a Packard Tri-Carb Scintillation Counter, model 3310. Controls of similarly prelabeled collagen gels without cells were incubated under identical conditions with 3 ml of each of the following: 0.9% NaCl; basal growth medium supplemented with 10% normal horse serum; and basal growth medium supplemented with 10% heat-inactivated horse serum.

Collagenolysis of reconstituted gels of prelabeled collagen also was carried out with the bacterial enzyme from *Clostridium histolyticum* (Nutritional Biochemicals Corp., Cleveland, Ohio). The incubation of 5 mg of the enzyme in a culture dish containing collagen gel and 3 ml of PBS for 16 hr at 37°C resulted in complete lysis. Following centrifugation at 20,000*g* for 1 hr, 1 ml of the supernatant was used for scintillation counting as described previously.

Staining of gels. In order to contrast areas of clearing, gels were fixed either with 10% formalin and stained with van Gieson's stain (9) or fixed with 2% glutaraldehyde and postfixed with 1% osmium tetroxide (10).

Protein and cell number determinations. Protein determinations were according to the Lowry method (7), and cell number estimations were made according to the procedure of Sanford *et al.* (8).

Radioisotopically labeled cellular proteins. The cell pellet following the washing procedure was solubilized in 0.5 ml of 2 *N* NaOH and prepared for scintillation counting according to the method of Mans and Novelli (11).

Histology. Collagen gels that were infiltrated with growing 2-31 cells were fixed in

10% formalin, dehydrated, embedded in paraffin, cross-sectioned at 7 μm, stained with hematoxylin and eosin, and examined with a light microscope.

Results. Upon subculture, the cells of line 2-31 in their 36th passage were observed to attach immediately to the gel surface with nearly 100% efficiency. Cell division was observed to commence within 2 hr after plating. Surface growth did not appear to be oriented along the longitudinal axis of the reconstituted collagen fibers. Instead, surface growth was radial at the periphery of the clones. However, some cells were observed to migrate to varying depths into the gel substrate where it was impossible to note the direction of growth in relation to any particular fiber or fibers within the gel substrate (Fig. 1). In the vicinity of the clones an immediate area of clearing was evident in contrast to the overall opalescence of the collagen gel. It should be pointed out that reconstituted collagen gels are often considered to be "clear" both on direct observation and on observation with the light microscope (4) but, nevertheless, they have an opalescence which can be seen when the gel is rotated slightly to an appropriate angle toward the incident light. As peripheral growth continued, the entire gel was rendered completely clear and incapable of fixing either stains of van Gieson or 1% osmium tetroxide (Fig. 2). However, the collagen gel remained rigid and retained its attachment to the culture dish.

After cell growth had led to the loss of opalescence in radioisotopically labeled collagen gels, the spent incubation medium removed from the culture dishes had the same level of radioactivity as the medium removed from control gels without any cell growth (Fig. 3).

Since human serum alpha-globulins have been shown to be potent inhibitors of human skin collagenase (12) it appeared likely that the alpha-globulins present in the horse serum supplement to the basal growth medium might have acted in a similar manner. Therefore, at the tenth day of culture 0.5% bovine serum albumin and 0.5% polyvinyl-

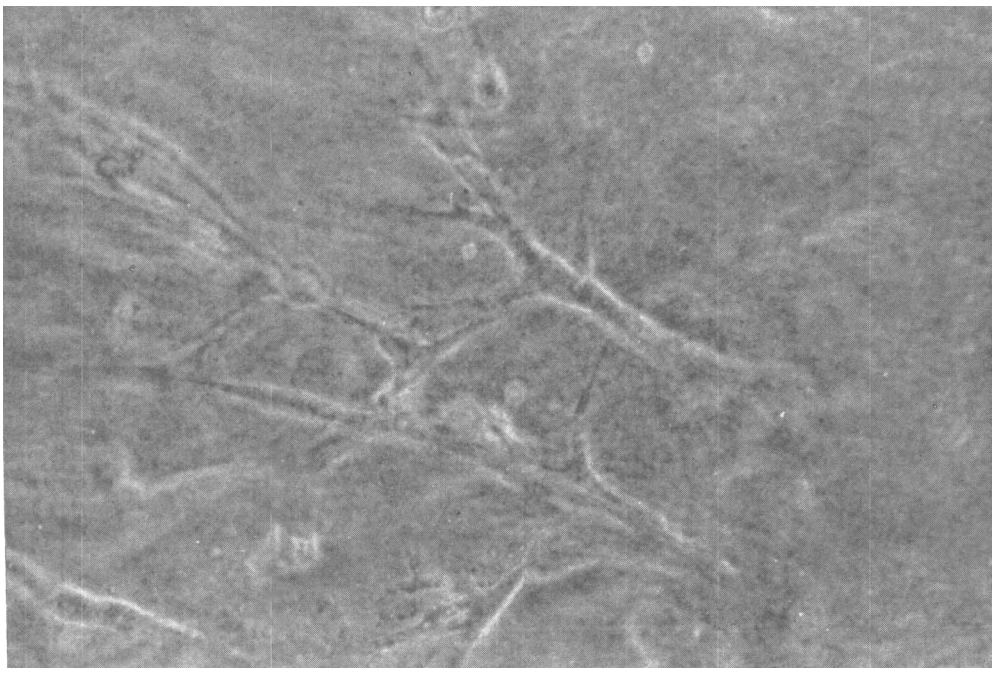

FIG. 1. Growth of 2-31 cells which had invaded the collagen gel to an approximate depth of 0.5 mm. Thickness of collagen gel was estimated to be 2.0 mm. ×600.

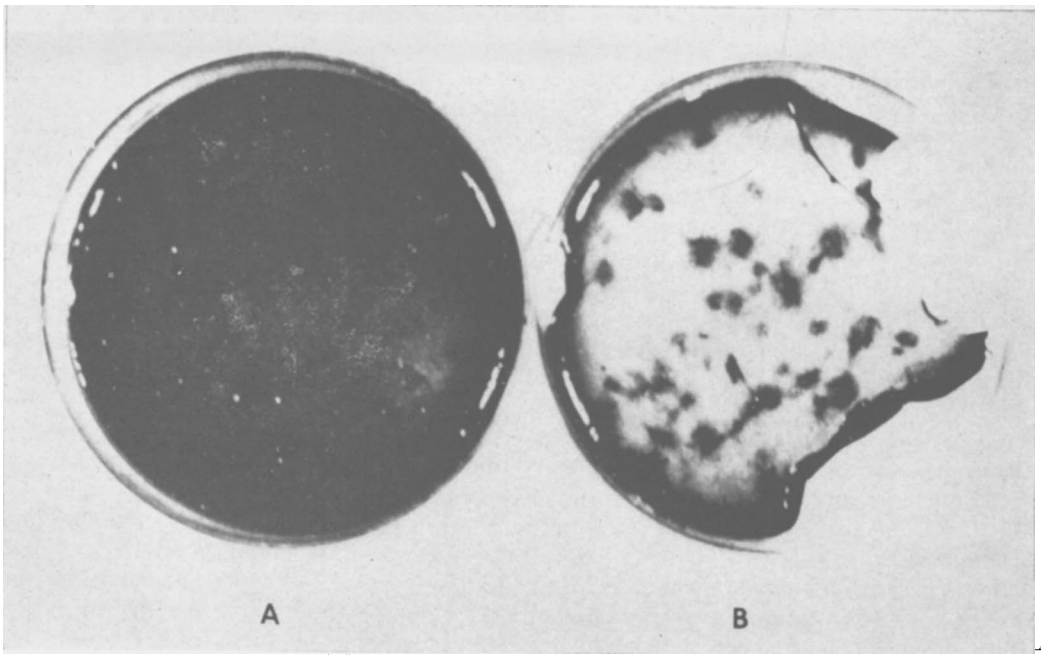

FIG. 2. A. Growth of 2-31 cells on collagen gels fixed with 2% glutaraldehyde and post-fixed with 1% OsO_4. Clearing 1 wk after subculture is becoming evident in the lower right quadrant. B. Generalized clearing of collagen gel at 4 wk. Areas of cell growth, very apparent at the periphery, exhibit osmophilia.

pyrrolidone (w/v of basal growth medium) were substituted for the serum supplement for a period of 1 wk (two complete changes of media). Radioactivity of the incubation medium did not increase significantly over control values following the substitution of the horse serum (Fig. 3).

A release of label was observed in the

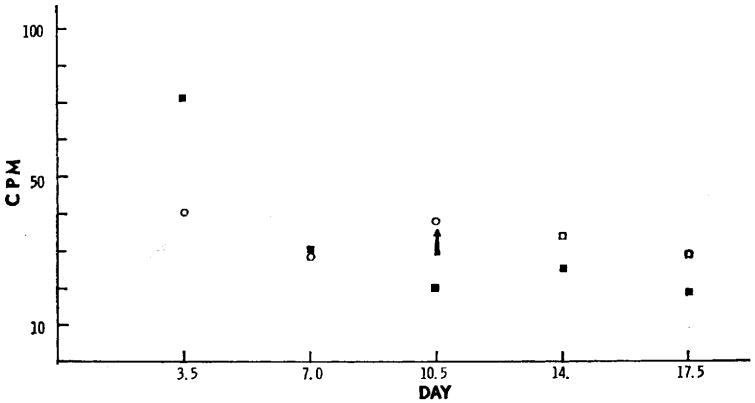

FIG. 3. Cpm/ml of spent medium representing release of ^{14}C-labeled soluble collagen peptides from reconstituted collagen gel culture dishes at each medium change (84 hr). (■) Control, collagen gel culture dish, minus cells (basal medium + 10% horse serum). (○) Same as (■) except that collagen gel served as a matrix for the growth of 2-31 cells. (↑) Substitution of horse serum with 0.5% PVP and 0.5% BSA as a supplement to the basal growth medium. (□) Same as (○) except horse serum replaced as supplement to the basal medium with 0.5% PVP and 0.5% BSA.

TABLE I. Labeled Control Gels Incubated with Bacterial Collagenase.

Incubation conditions of gel	Counts per minute		
	^{14}C-incorporation/mg cell protein	Total accumulated radioactivity in spent medium	Radioactivity of lysed collagen gel
1. 2-31 cells + basal growth medium + 10% normal horse serum	366	504	6185
2. Control: No cells, gels incubated with basal growth medium + 10% normal horse serum	—	498	5973

These values represent sample collagen gels.

Approximately 2 ml of collagen were dispensed to each culture dish prior to heat gelation with an estimated radioactivity of approximately 8000 cpm. See Discussion.

medium used for the equilibration of the gels described in Methods and Materials. The total radioactivity present in the growth medium following 24 hr of equilibration averaged 498 cpm. Equilibration with either 0.9% NaCl or with growth medium supplemented with 10% heat inactivated horse serum released 336 and 360 cpm, respectively. Therefore the observed release of radioactive label into the medium was unrelated to the presence of growing cells and the composition of the equilibration medium.

Cells removed from cleared radioisotopically labeled collagen gels were found to incorporate an average of 366 cpm/mg into TCA precipitable cellular proteins. Subsequent incubation of the preceding gels with bacterial collagenase resulted in complete lysis and the release of 6185 cpm as

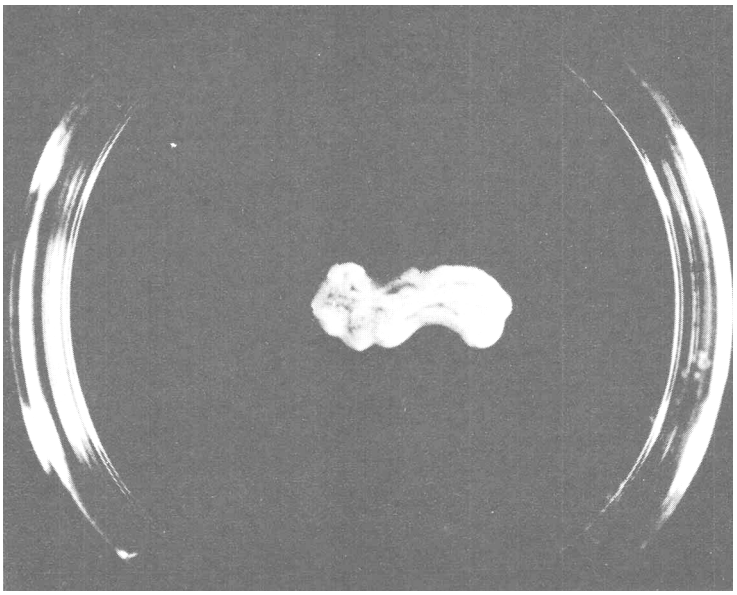

FIG. 4. Condensation of collagen gel by 2-31 cells at 8 wk.

soluble peptides into the incubation medium. Labeled control gels incubated with bacterial collagenase under identical conditions but without cells gave similar values (Table I).

Of special interest to us was the fate of the collagen gel when cellular growth was permitted to proceed for periods up to 12 wk. At about the fourth to fifth week the surface cellular growth became very dense and consisted of as many as four layers of cells. The migration of cells throughout the collagen gel matrix was so pronounced that some cells grew on the bottom of the culture dish under the gel. Detachment of the gel first occurred at the side of the culture dish and was followed by a gradual detachment from the bottom. The net result appeared to be a gentle rolling up of the edges of the gel which culminated in the total detachment and condensation of the gel into a tight, compact mass (Fig. 4).

Specimens of condensed gel prepared for histological examination showed fibroblastic cells at the surface which in some areas were arranged in as many as four layers of cells. Radiating toward the center of the specimen were long, spindle-shaped cells whose direction appeared to be oriented along weakly staining eosinophilic fibers, presumed to be the reconstituted collagen. In areas where convolutions of the collagen gel had occurred, the cells appeared highly pleomorphic with marked variations of nuclear size, shape and staining intensity. These sections gave the general impression that the cells were viable and actively growing (Fig. 5).

In contrast to the 2-31 cells, the propagation of "L" cells and HeLa cells on collagen gel surfaces did not result in invasion, clearing, or condensation of the collagen gel.

Discussion. The results of these investigations offer evidence that the alteration in the light transmission and staining properties of reconstituted collagen gels observed in the immediate vicinity of growing clones of cells from culture 2-31 cannot be attributed to collagenolysis.

The release of labeled soluble collagen

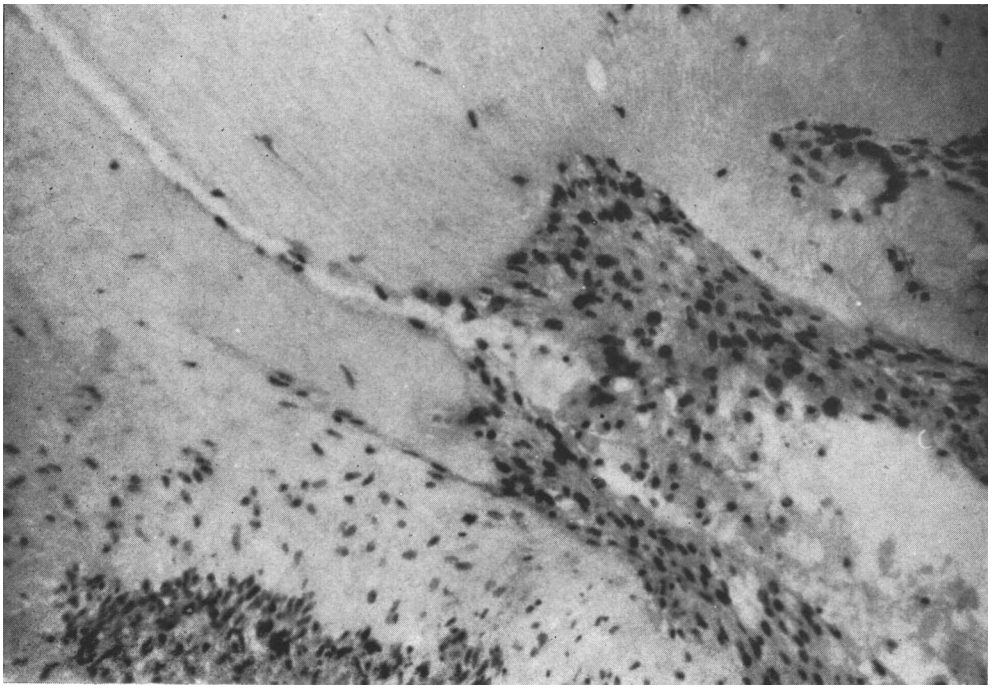

FIG. 5. Histological cross section through the specimen in Fig. 4. Details appear in text. ×250.

peptides is therefore judged to be a more reliable method than clearing for the purpose of demonstrating collagenolysis by cells growing on prelabeled collagen gel surfaces. However, as demonstrated by the controls to these experiments, at least 1000 cpm of labeled peptides can be released without collagenolysis into the medium during the equilibration process and subsequent incubation period. It is believed that these counts represent soluble collagen peptides which had not taken part in the heat gelation process and in addition may represent other labeled proteins which were isolated by the fractionation procedure. It is likely that these had served as the source for the subsequent uptake of radioactivity into cellular proteins. The total incorporation of radioactivity into cellular proteins may have been greater than demonstrated since trypsinization was used in effecting the detachment of cells from the gel surface. Dalen and Todd (13) have shown that cytoplasmic processes remain subsequent to cellular detachment with trypsin.

Although the intention of these experiments was not to determine the mechanism responsible for the "clearing" of the gel, it certainly has been shown that although clearing is cell mediated it is not due to collagenolysis.

What may prove to be of far greater consequence is the observed behavior of cells of this culture when grown on firm gels of collagen. Although a cell monolayer was established on the gel surface there was also a unique migration by cells of this culture into the gel matrix. As proliferation continued within the gel matrix, variations in cell morphology were observed. Whether the observed pleomorphism represents differentiation or is related to differences in the nutritional microenvironment remains of particular interest since cells of this culture are believed to possess pluripotentiality (1).

Summary. A previously reported (1) hamster monolayer cell culture derived from a fibro-hemangiosarcoma was grown on firm gels of reconstituted collagen. The gel was cleared without concomitant collagenolysis as judged by the release of labeled soluble collagen peptides from the reconstituted gel. In the final phase, the collagen was condensed. Histological examination of the condensed gel revealed a proliferation of cells showing variations in cell appearance within the gel matrix.

1. Cuprak, L. J., and Lever, W. F., Proc. Soc. Exp. Biol. Med. **141**, 494 (1972).

2. Taylor, A. C., Levy, B. M., and Simpson, J. W., Nature (London) **228**, 366 (1970).

3. Eisen, A. Z., and Gross, J., J. Develop. Biol. **12**, 408 (1965).

4. Ehrmann, R. L., and Gey, G. O., J. Nat. Cancer Inst. **16**, 1375 (1956).

5. Hillis, W. D., and Bang, F. B., Exp. Cell Res. **26**, 9 (1962).

6. Berman, M. B., Manabe, R., and Davison, P. F., Anal. Biochem. **54**, 522 (1973).

7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

8. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, J. E., J. Nat. Cancer Inst. **11**, 773 (1951).

9. Fullmer, H. M., and Gibson, W., Nature (London) **209**, 728 (1966).

10. Pease, D. C., "Histological Techniques for Electron Microscopy," 2nd ed., p. 39. Academic Press, New York (1964).

11. Mans, R. J., and Novelli, G. D., Arch. Biochim. **94**, 48 (1961).

12. Eisen, A. Z., Bauer, E. A., and Jeffrey, J. J., Proc. Nat. Acad. Sci. USA **68**, 248 (1971).

13. Dalen, H., and Todd, P. W., Exp. Cell Res. **66**, 353 (1971).

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