*SYNTHESIS OF COLLAGEN AND HYALURONIC ACID BY FIBROBLAST HYBRIDS**

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Very little is known about the mechanisms of control of differentiated functions in mammalian cells. The present work, concerned with the synthesis of collagen and hyaluronic acid, was undertaken with the aim of obtaining information on the control of these two differentiated functions in the fibroblast. Established cell lines of fibroblastic origin differ greatly in their ability to synthesize these substances.[1, 2] The use of the technique of somatic cell hybridization (review in ref. 3) enabled us to combine in a single cell the genomes of two fibroblasts differing in the rates of production of collagen and hyaluronic acid, and to study the dominance relationships between the genes involved in these functions.

Materials and Methods.—The hybrid clones studied were produced in two crosses between a single line of fibroblasts synthesizing considerable amounts of collagen and hyaluronic acid, and two different fibroblast lines producing very little collagen and no detectable amounts of hyaluronic acid.

The "parental" line common to the two crosses is 3T6 clone 7, isolated from 3T6, an established mouse cell line.[4] Clone 7 synthesizes slightly smaller amounts of collagen and hyaluronic acid than the parent population 3T6 (cf. refs. 1 and 2).

The two established cell lines producing very little collagen and no hyaluronic acid are NCTC 2555 and NCTC 2472[5] clone 6,[6] previously shown to undergo hybridization.[7]

The karyological features of the parental lines used for the identification of hybrids of the two crosses (3T6-7 × 2555 and 3T6-7 × 2472-6) are as follows. The cells of line 3T6-7 contain among their 70–79 telocentric chromosomes (mode 73), 2–3 distinct "markers" recognizable in hybrids of both crosses: one medium-sized telocentric with a pronounced proximal secondary constriction, and one or two small telocentrics with a very strong median constriction appearing as a feebly staining "heterochromatic" gap. Cells of line 2555 (54–58 chromosomes, mode 57) are characterized by the presence of 17–20 biarmed chromosomes and the absence of "rabbit-ear" chromosomes, of which several are observed in normal mouse cells and in 3T6. Thus, hybrids of the cross 3T6-7 × 2555 are identified by the simultaneous presence of the two or three "markers" and the "rabbit-ear" chromosomes of line 3T6-7, and of the biarmed chromosomes contributed by line 2555.

Cells of line 2472-6 contain 48–53 (mode 50) chromosomes, among them one extra-long telocentric and one biarmed chromosome. The hybrids of the cross 3T6-7 × 2472-6 were therefore easily identified by the presence of the 3T6-7 "markers" and of the extra-long and the biarmed chromosome contributed by 2472-6.

Cultures of all cell lines were grown in the Dulbecco-Vogt modification of Eagle's medium, containing 10% calf serum. Ascorbic acid (50 μg/ml) was added to the medium at least several days before measurements of collagen synthesis.[8]

For hybridization, mixed cultures of the two cell lines to be crossed were grown at 29°C.[6] Samples of these cultures were periodically examined karyologically for the presence of hybrids. When the proportions of hybrid cells reached 20% in cross 3T6-7 × 2555 and 90% in cross 3T6-7 × 2472-6, the mixed cultures were cloned at 36°C. Among the clones isolated, three from each cross were kept for further investigation.[9] An uncloned hybrid population, resulting from the overgrowth of the mixed culture 3T6-7 × 2555 by the hybrid cells, was also available. The purity

TABLE 1

COLLAGEN SYNTHESIS BY PARENTS AND HYBRIDS ($\Delta C/\Delta P$)

Parents		Hybrids	
3T6-7	3.8		
		Clone 1	2.23
		Clone 2	1.90
		Clone 4	0.95
2472-6	0.49		
	Mean = 2.1		Mean = 1.69
3T6-7	3.8		
		Clone 1	2.42
		Clone 2	3.67
		Clone 7	2.87
2555	0.21		
	Mean = 2.0		Mean = 2.99

TABLE 2

HYALURONATE PRODUCTION BY PARENTS AND HYBRIDS ($\mu\mu$G/CELL/DAY)

Parents		Hybrids	
3T6-7	6.21		
		Uncloned population	1.24
		Clone 1	0.81
		Clone 4	0.83
2472-6	0		
3T6-7	6.21		
		Clone 1	4.3
		Clone 2	3.3
		Clone 7	4.1
2555	0		

of all hybrid cell lines used for determinations of collagen and hyaluronic acid production was checked karyologically. At the time these determinations were performed, the different hybrid clones had undergone a minimum of 30–50 cell generations since their isolation. Therefore, all macromolecules synthesized by the parental cells prior to hybridization were essentially diluted out.

Measurements of the rate of collagen synthesis were carried out in dense, essentially stationary populations by exposing them to C^{14}-L-proline and comparing the radioactivity of the proline and hydroxyproline incorporated into the culture protein.[10] From this ratio and the known abundance of hydroxyproline in collagen and of proline in the cell protein, the differential rate of collagen synthesis with respect to the synthesis of other proteins was calculated.[10] In stationary fibroblast cultures this value appears not to be affected by changes in the over-all rate of protein synthesis[11] and is therefore the most significant measure of differentiated function in this cell type.

Hyaluronate measurements were carried out as described previously[2] on medium which had been in contact with a dense cell layer for 24 hr. The total amount of hyaluronate present in the medium was corrected for the number of cells present, as determined by counting a trypsinized suspension made from the cell layer, and the results are expressed as $\mu\mu$g produced per cell per day.

Results and Discussion.—The results obtained are shown in Tables 1 and 2. All hybrids synthesize measurable amounts of both collagen and hyaluronic acid. For both substances, the values obtained for the hybrids are intermediate between those of the two parents.

In the case of collagen, we are dealing with a protein whose differential rate of synthesis is measured with respect to that of all other cell proteins. If each genome functions in the hybrid as it did in the parent cell, the $\Delta C/\Delta P$ value given by the hybrid should be equal to the average of the values of its parents. Most of the values obtained for both types of hybrids approximate this expectation, indicating that there is no dominance of the characteristics of one of the parents over those of the other. However, it will be noticed that all values given by the hybrid

clones from the cross of 3T6-7 × 2555 are somewhat higher than expected, and that, while two hybrid clones from the cross 3T6-7 × 2472-6 are close to the average of the parental values, a third one is definitely below it. The interclonal variation in this cross does not obscure the difference between the hybrids of the two crosses. However, differences of this magnitude could easily arise from unequal representation of parent DNA's in the hybrids as (1) the contribution of DNA by each of the parents is probably not equal, especially since hybrids may have been formed by fusion of nonmodal cells, and (2) total chromosome counts show that the hybrids must have lost some of the chromosomes of their original complement (cf. ref. 12) by the time their synthetic activities were measured.

In the case of hyaluronic acid, we are dealing with a more remote effect of gene function, namely, with the synthesis of a polysaccharide by an enzyme,[13] and the rate of synthesis of the polysaccharide need not be determined exclusively by the rate of synthesis of the enzyme. However, the values obtained for hyaluronate synthesis by hybrids are, like those for collagen, intermediate between the parental values and are again different for hybrids of the two crosses, those containing the 2555 genome giving higher values than those with the 2472-6 genome.

Since the parent cells are all from established lines which undoubtedly have undergone some alterations in control mechanisms during their culture history, it would be unwarranted to draw, from the present evidence, conclusions about the mechanisms of control of differentiated functions in mammalian cells in general. However, it may be concluded (1) that the genes of 3T6-7 which are responsible for collagen and hyaluronate synthesis do function in hybrids containing another genome which expresses these functions very poorly or not at all in the corresponding parent cell, and (2) that the activities of the 2472-6 and 2555 genes responsible for these functions do not reach, in the hybrid cells, the level of the corresponding 3T6-7 genes. In fact, apart from the systematic difference between the hybrids of the two crosses (which, as suggested above, probably has a trivial explanation), there is no indication of interaction of the two parental genomes in the hybrids. It remains to be seen whether these facts reflect the properties of control mechanisms in diploid mammalian cells or whether they are due to genetic changes (analogous, for example, to operator mutations in bacterial cells[14]) which may have occurred in the cells of the established lines used in these experiments.

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[1] Green, H., and B. Goldberg, these PROCEEDINGS, **53**, 1360 (1965).

[2] Hammerman, D., G. J. Todaro, and H. Green, *Biochim. Biophys. Acta*, **101**, 343 (1965).

[3] Ephrussi, B., and S. Sorieul, *Univ. Michigan Med. Bull.*, **28**, 347 (1962).

[4] Todaro, G. J., and H. Green, *J. Cell Biol.*, **17**, 299 (1963).

[5] Sanford, K. K., G. D. Likely, and W. R. Earle, *J. Natl. Cancer Inst.*, **15**, 215 (1954).

[6] Scaletta, L. J., and B. Ephrussi, *Nature*, **205**, 1169 (1965).

[7] Barski, G., S. Sorieul, and F. Cornefert, *Compt. Rend.*, **251**, 1825 (1960).

[8] Green, H., G. J. Todaro, and B. Goldberg, *Nature*, in press.

[9] The design of these hybridization experiments does not permit us to say with certainty that the different hybrid clones isolated from each cross represent the progeny of multiple rather than of single matings. However, the former appears more likely.

[10] Green, H., and B. Goldberg, *Nature*, **204**, 347 (1964).
[11] Green, H., and B. Goldberg, in preparation.
[12] Ephrussi, B., L. J. Scaletta, M. A. Stenchever, and M. C. Yoshida, in *Cytogenetics of Cells in Culture*, Symposia of the International Society for Cell Biology, vol. 3 (1964), p. 13.
[13] Schiller, S., G. A. Glover, and A. Dorfman, *Biochem. Biophys. Res. Commun.*, **5**, 344 (1961).
[14] Jacob, F., and J. Monod, *Bull. Soc. Chim. Biol.*, **46**, 1499 (1964).

*DOUBLE-STRANDEDNESS OF MEIOTIC PROPHASE CHROMATIDS TO LIGHT MICROSCOPE OPTICS AND ITS RELATIONSHIP TO GENETIC RECOMBINATION**

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During the course of recombination studies involving temperature treatment to maize microsporocytes, a striking effect on chromosome organization at diplotene and diakinesis was observed. Cells fixed at these stages, which had been treated earlier as described below, characteristically contained ten bivalents each with the appearance of a loose collection of tangled twine with no knobs or centromeres discernible (Fig. 1). In such treated cells fixed at diakinesis, chromatids could be seen to be composed of two apparently independently coiled longitudinal strands. The components of an occasional bivalent were so spread and flattened in preparation that each of the four chromatids could be seen clearly to be made up of two half chromatids (Fig. 2). This report was submitted because the degree of strandedness of chromatids is a subject of continuing controversy with important bearing on the theory of crossover mechanism and because light photomicrographs which illustrate chromatid doubleness at first meiotic prophase have not been previously published, to the writer's knowledge.

Methods and Results.—Maize plants (*Zea mays* L.) were grown in a plant growth chamber at a constant temperature of 25°C and a relative humidity of 80 per cent with a diurnal light period of 14 hr. Artificial light was provided by a mixture of cool-white fluorescent and incandescent lamps.

At sporocyte stage, plants to be treated and sister control plants were removed from the growth chamber to a room at approximately 25°C. Stalks of both were opened, young tassels (attached to their stalks) carefully spread on a Lucite plate supported by a ring stand, and alternate spikelets were collected serially from a number of marked branches. The collected spikelets were stored in a freezer individually in vials containing alcohol-acetic 3:1 mixture (for later classification for stage of meiotic advancement). Remaining tassel material of both treatment and control plants was then carefully returned to its stalk, thermometers were inserted, and the incisions bandaged. At this time tassel regions of treatment plants were wrapped with two layers of turns of a hose through which water was circulated at 36°C (under the control of a Haake ultrathermostat). By this method the temperature of the microsporocytes was raised (at an approximately constant rate) to nearly 36°C in 25 min and maintained at this level for 155 min.