*CHRONOLOGY AND PATTERN OF HUMAN CHROMOSOME REPLICATION, IV. AUTORADIOGRAPHIC STUDIES OF BINUCLEATE CELLS**

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The chronology and pattern of chromosomal DNA replication in mammalian cells, including human, have been studied almost exclusively in uninucleated cells. Except for some data recently obtained in our laboratory on human tetraploid cells,[1] we are unaware of reports dealing with the sequence of DNA duplication of chromosomes in binucleate cells. Asynchrony of chromosomal DNA replication in various diploid human cell lines has been demonstrated by a number of workers using autoradiographic techniques.[2–10] A recent publication suggests that all chromosomes appear to finish DNA replication at the same time, but approach that end at different rates, with one of the X chromosomes in the normal female replicating much more rapidy than the other chromosomes during the last few hours of the *S*-period.[11] A similar pattern has been observed in our laboratory for tetraploid cells.[1] Most important, almost all of the chromosomes of a given group of homologues of a tetraploid cell were labeled to some degree when the tritiated thymidine was "pulsed" for 10 min, 4–6 hr before fixation. In these cells, marked labeling of all four homologues was evident when the isotope was in contact with cultured cells for 2 hr. The availability of a human diploid cell line with an impressive number (1–4%) of binucleate cells offered the unusual opportunity of examining the chronology of chromosomal replication in the two nuclei. The study indicates that one nucleus replicated its DNA considerably ahead of the other, and then awaited completion of DNA replication in its mate before going into metaphase.

Materials and Methods.—The cell line studied (RPMI #8205, MC #2) was derived from the blood of a 54-year-old woman with acute myeloblastic leukemia. The frequency of binucleate cells in the cultured line varied from 1.3% to nearly 4%. Cells were considered to be binucleate when surrounded by one cellular membrane and containing two distinct nuclei in a common cytoplasm. This cell line was cultured originally in McCoy's medium with 20% calf serum and 0.2% human albumin. Subsequently, the cells used in the present studies were cultured in suspension in RPMI media,[12] usually in 16-oz bottles made by Brockway. Details regarding the cell line will appear elsewhere.[13] Tritiated thymidine (specific activity 6.7 c/mM) was added to the culture (final concentration of 1 μc/ml) for periods of time ranging from 20 min to 5 hr before fixation and left in the culture for the total interval of time unless pulse experiments are indicated. Most of the details regarding the processing of the cells in preparation for chromosome analysis and for autoradiography have been described in previous publications.[9, 10] Colchicine (1 μg/ml of medium) was present in the culture medium during the last 2 hr of incubation. At the end of incubation the culture was transferred to centrifuge tubes, and the cells were packed by centrifugation. The supernatant was discarded and the cells were then washed several times with Hank's solution, followed by treatment with hypotonic solution in order to swell the cells prior to spreading of the chromosomes. The material was then fixed in acetic-alcohol (1:3), and slides were prepared by an air-drying technique and stained with acetic orcein. These techniques cause little breakage and maintain cellular integrity as ascertained by the persistence of the cytoplasm and an intact cellular membrane surrounding the metaphase chromosomes. Under these conditions the metaphase chromosomes of two contiguous cells can be easily differentiated by their morphological appear-

ance, depending on the stage of metaphase at which fixation occurred. Such differentiation is readily possible even when chromosomes of two metaphases are admixed. Slight differences in timing between cells in metaphase would yield chromosomes of distinctly different lengths and dissimilar appearance, making identification of chromosomes of different cells rather facile. The above points have been made in order to stress the origin of the metaphase chromosomes described in this paper as from the same cell (i.e., binucleate) as evidenced by (1) identical morphologic appearance of chromosomes, (2) presence of cell membrane around the metaphases, and (3) the relatively high incidence of binucleate cells and metaphases with 92 chromosomes. The term tetraploid will be used to designate cells with 92 chromosomes contained within one nucleus, whereas the binucleate cell also contains 92 chromosomes but has two distinct nuclei.

Results.—Examination of the smears of the cultured cells revealed the presence of binucleate cells (1–4%). The cell line was shown to be diploid with a sharp mode of 46 chromosomes in 72 per cent of the uninuclear cells (Table 1). Autoradiographic studies of the diploid cells revealed a late-labeling X chromosome and additional late-labeling elements in groups *B*, *D*, and *E* (Fig. 1), as has been described for other diploid human cell.[2–10]

In the initial studies of this cell line the H^3-thymidine was added 2–5 hr before fixation for a 10-min interval, in an attempt to "pulse-label" the chromosomes. It was observed that although the number of grains over the chromosomes was scant, the grains were confined to only two chromosomes of each homologous group in most cells containing the tetraploid or near-tetraploid number of chromosomes. This became more evident when the tritiated thymidine was added to the culture 3–7 hr before fixation. A most striking finding was that the grains were distributed over only one half of the chromosomes in the tetraploid metaphases (Fig. 2), and only two chromosomes in each homologous group were labeled (Figs. 3 and 4).

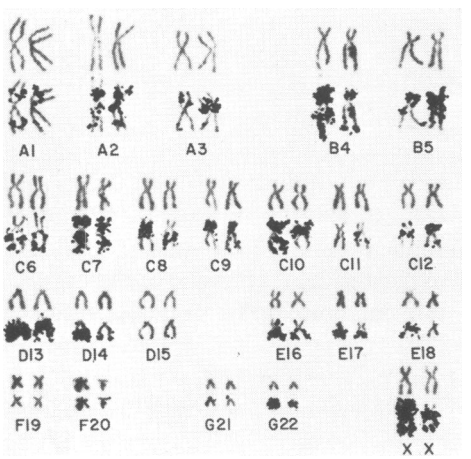

FIG. 1.—Karyotypes, before and after autoradiography, of a diploid cell from the line used in the present study. Tritiated thymidine was added 5 hr before fixation of the cells. Late labeling of one *X*, *B*, and *D* group chromosomes is shown.

In order to intensify the incorporation of the H^3-thymidine with resultant higher grain content of the chromosomes, the cells were exposed to the tritiated deoxyriboside continuously for the last 5 hr of culture. This approach allowed heavy labeling

TABLE 1

THE DISTRIBUTION OF CHROMOSOME NUMBER AND FREQUENCY OF POLYPLOID CELLS

	Chromosome Number					
	42	*43*	*44*	*45*	*46*	Total
Number of cells	1	2	10	25	99	137
	s	*2s**	Endoreduplicated			Total
Number of cells	267	16		1		284

s = Cells with a diploid range of chromosome number.
2s = cells with a tetraploid range of chromosome number.
* This material contained about 2% of binucleate cells. Hence, it is probable that the remaining cells with the *2s* number of chromosomes (about 4%) were tetraploid.

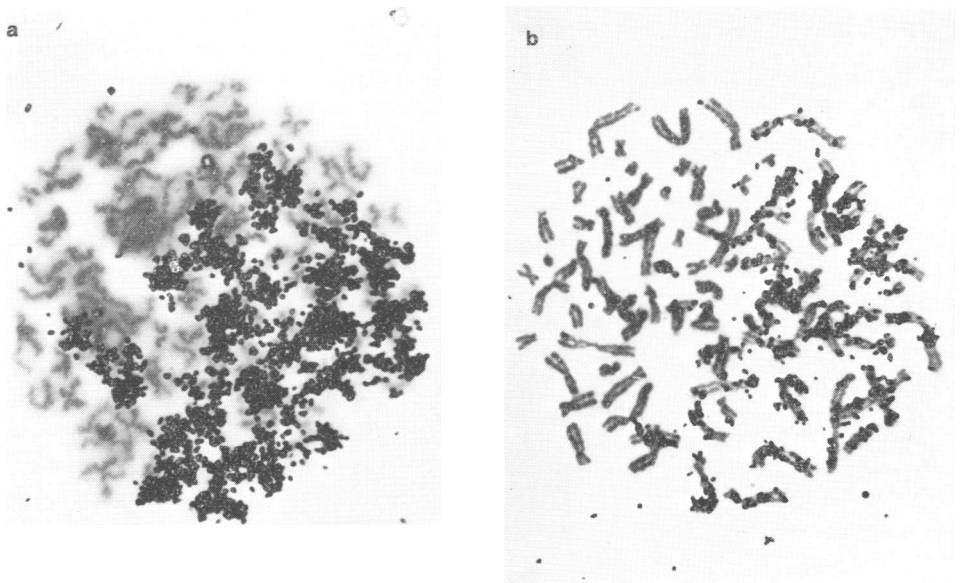

FIG. 2.—Autoradiographs of two metaphases with a tetraploid number of chromosomes of cultured human cell line RPMI#8205. The thymidine was present in the culture medium during the last 5 hr of incubation. The grain distribution is primarily confined to one half of each metaphase. The cytoplasm surrounding each metaphase is not visible becuase of the photographic technique, but was observed during microscopy. The similar appearance of the chromosomes, the shape of the metaphases, and the karyotypes of the metaphases all point to the origin of the chromosomes from individual binucleate cells.

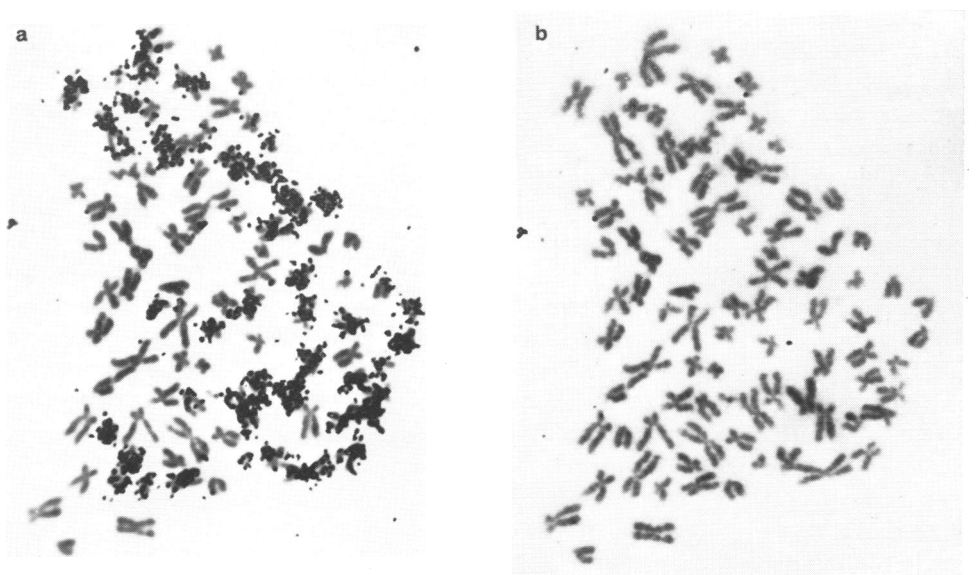

FIG. 3.—Metaphases with 92 chromosomes before and after autoradiography, obtained under conditions similar to those described in Figs. 1 and 2*a*. The autoradiograph of (*b*) reveals only one half of the total number of chromosomes to be labeled.

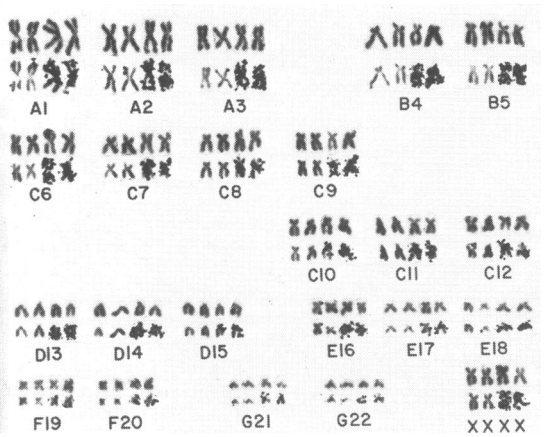

FIG. 4.—Karyotypes, before and after autoradiography, of the metaphases in Fig. 3. Only two chromosomes are labeled in each group of homologues, strongly indicating the binucleate origin of the chromosomes, the labeled homologues coming from one nucleus and the unlabeled from another.

of the chromosomes and interphase nuclei and left little doubt that the chromosomes of only one of the two nuclei in binucleate cells were labeled. In some cases, two of the four homologues were very heavily labeled, whereas the other two were only slightly labeled, indicative that tritiated thymidine was added at a time when DNA replication was very active in one nucleus and nearing completion in the other nucleus. Examination of interphase nuclei confirmed this impression, since in 10 per cent of binucleate cells either only one of the nuclei contained label or, if both were labeled, the intensity of the labeling was greatly divergent in the two. A substantial number (0.1%) of these cells contained one labeled and one unlabeled nucleus even when the H^3-thymidine was added as late as 20 min before fixation (Fig. 5). Asynchronous replication was also encountered in binucleate cells exposed for 5 hr to the tritiated thymidine. The karyotype of such a cell in metaphase is shown in Figure 4. In most of the labeled cells both nuclei appeared to be labeled equally, indicative of active DNA replication with overlapping *S*-periods in the two nuclei.

Discussion.—The presence of binucleate cells in the material studied, the total lack of labeling of one half of the total number of chromosomes in some of the cells exposed to tritiated thymidine for 5 hr, indicate that we are, indeed, studying the chromosomal replication of binucleate cells. It should be stressed that even with "pulse-labeling" of tetraploid cells, incorporation of the tritiated thymidine occurs into all chromosomes to a varying degree.[1] Furthermore, when such cells are exposed to thymidine for 5 hr, incorporation of the precursor into all chromosomes is evident.

The present study elucidates the pattern of chromosomal DNA replication in human binucleate cells. Apparently, DNA replication in one nucleus is completed long before its companion; however, only upon subsequent completion of DNA replication in the second nucleus will the cell proceed to metaphase. This indicates that the G_2-period of one nucleus is substantially longer than that of its companion. If it is assumed that the *S*-periods of the nuclei are of equal length, then the duration of the G_1-periods is affected to the same extent to which the G_2-periods differ, e.g., the nucleus with the longer G_2-period has a comparably shorter G_1, and vice versa. Should one nucleus enter metaphase prior to its mate, not only would the morphologic appearance of the chromosomes be strikingly different, but also one would expect labeling of some of the chromosomes to occur during the 5-hr period in such a nucleus. In addition, one would expect to observe both a division figure and interphase nucleus in some of the cells. To date, no cells of this type

have been seen upon examination of 100,000 cells of the line studied. Thus, the data are best interpreted as indicative of asynchronous completion of DNA replication in the two nuclei of a binucleate cell. Upon completion of DNA replication in both nuclei, synchronization of metaphase appears to occur.

In order to ascertain the fate of the binucleate cells, a thorough search was made for the number and orientation of the spindles in telophase cells. Unfortunately, the search has not yielded definitive information, although it is our opinion that most of the binucleate cells divide into two tetraploid ones. Some support for this statement can be obtained from Table 1 which shows that the number of tetraploid cells exceeded that of the binucleate ones by a factor of 2.

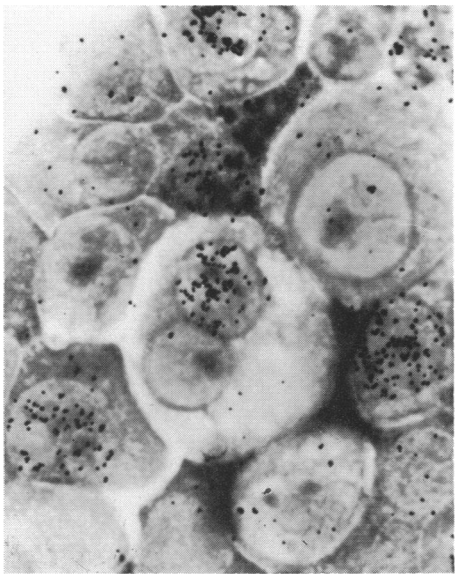

FIG. 5.—Photograph of a binucleate cell (stained with Giemsa) showing the labeling of only one nucleus when the cell was exposed to tritiated thymidine during the last 20 min of incubation. More intense labeling of one nucleus was obtained when the thymidine was present in the medium for longer periods of time. The finding indicates that one nucleus replicates its DNA asynchronously with that of the other nucleus.

The authors are unaware of investigations on mammalian cells exactly comparable to the present study. In cultured human leukocytes and in direct bone marrow preparations, metaphases with a tetraploid (or higher) number of chromosomes are not infrequently observed.[14, 15] Particularly in marrow, such metaphases may constitute 1–2 per cent of the total cell population, and these tetraploid, octoploid, and higher ploidy cells are thought to originate, most likely, from multinucleated megakaryocytes, plasma cells, or normoblasts. It is of interest that the chromosomes within a given metaphase are very similar in appearance, as was observed in the cell line of the present study, whereas the length and chromatid morphology may differ from one hyperploid metaphase to another. It is hoped that investigations of bone marrow material containing megakaryocytes will shed further light on the DNA replication of multinucleated cells.

Stubblefield[16] artificially induced the formation of multinucleated cells with colcemid in cultured diploid Chinese hamster cells, the multiple abnormal nuclei being due to random conglomeration of different chromosomes into nuclei of various sizes and compositions. Autoradiography revealed these various-sized nuclei to undergo asynchronous DNA replication. Since each nucleus of the Chinese hamster multinucleate cells does not contain the same chromosome number or constitution and the nuclei were abnormal in several respects, the results may not be analogous to those obtained with the binucleate human cells in which each nucleus is diploid.

Studies of binucleate protozoa may bear some relationship to the data presented. Kimball and Prescott[17] indicate that the two macronuclei in double animals of *Euplotes* begin their replication simultaneously. This conclusion was based on the

distance traversed by the bands along the nuclei. The use of such migration as a reliable criterion of the synchrony of DNA synthesis in two separate nuclei awaits confirmation by H^3-thymidine autoradiography. If the above findings are confirmed, they would differ from those presented for the mammalian cells studied by us. On the other hand, Prescott *et al.*[18] have shown that micronuclear and macronuclear DNA synthesis in the same organism occur asynchronously. A similar finding has been reported by McDonald in *Tetrahymena pyriformis*.[19] Thus, the observations in micro- and macronuclei appear to be akin to those in our mammalian cell line. In this connection it is interesting that Zeuthen has reported that synchronized tetrahymena cells replicate their DNA asynchronously but go into mitosis at the same time.[20] Whether the factors operating in a cell population, which control the separate cycles of DNA synthesis and mitosis, are similar to those operative intracellularly in binucleate or multinucleated cells is an intriguing problem. At present almost nothing is known about the intracellular factors which control DNA synthesis in binucleate cells and which synchronize the mitotic events occurring in such a cell.

Summary.—Autoradiographic studies of a human cell line containing binucleate cells revealed asynchrony of DNA synthesis in one nucleus as compared to that of its mate. When tritiated thymidine was present in the culture during the final 5 hr of incubation, only two of the four homologues in some metaphases with 92 chromosomes were heavily labeled, while the other two were devoid of labeling. A plausible explanation of this observation is the completion of DNA synthesis in one nucleus substantially prior to that of its companion nucleus. However, after completing their DNA replication, the two nuclei in a binucleate cell appear to go into metaphase simultaneously.

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