Patterns of Initiation of DNA Replication in Cultured Cells from Human Females*

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Variations in the rate of incorporation of H³-thymidine along the salivary gland chromosomes of *Rhynchosciara* were observed by Ficq and Pavan (1957) and, through quantitative measurements, a disproportionate synthesis of DNA in a polytene chromosome region from the same organism was detected by Rudkin and Corlette (1957). The existence of a regular sequential mode of replication of chromosomal DNA was first observed by Taylor (1958) in the root tips of *Crepis* and a precisely synchronized pattern of DNA synthesis was found later in the macronucleus of a ciliated protozoan, *Euplotes* (Gall, 1959). In *Melanophus* and *Secale* chromosomes the heterochromatin was found to replicate at different times from those of euchromatin (Lima-de-Faria, 1959). More recently, asynchronous replication of chromosomal DNA has been demonstrated in cultured cells from the Chinese hamster (Taylor, 1960), in root tips of *Tradescantia* (Wimber, 1961), in HeLa S₃ cells (Painter, 1961), in cultured normal human cells (Lima-de-Faria, Reitaluc, and Bergman, 1961; German, 1962; Morishima, Grumbach, and Taylor, 1962; Gilbert, Muldal, Lajtha, and Rowley, 1962; Atkins, Taft, and Dalal, 1962; Moorhead and Defendi, 1963; Rowley, Muldal, Gilbert, Lajtha, Lindsten, Fraccaro, and Kaijser, 1963; Bader, Miller, and Mukherjee, 1963; Schmid, 1963; Mukherjee, Miller, Breg, and Bader, 1964), and in cells from *Bos taurus* (Mukherjee and Sinha, 1963; Gartler and Burt, 1964). All these studies have indicated overwhelmingly that the asynchronous pattern of DNA replication in any given complement is not random and that each chromosome follows a characteristically regulated system of duplication.

In most of the published autoradiographic studies on the duplication of human chromosomes, the pattern of termination of DNA replication was used

as a means of determining asynchrony; very little is known about the mode of its initiation in individual chromosomes of a complement. In his study on replication of DNA in HeLa S₃ cells, Painter (1961) has indicated that onset of DNA synthesis in all chromosomes is simultaneous, and this conclusion has been supported by Stubblefield and Mueller (1962); but German (1962) and Kikuchi and Sandberg (1964) observed that in cultured leucocytes from normal human males and females the process of DNA synthesis did not begin simultaneously in all chromosomes. The latter study was carried out by exposing the cultured leucocytes for 10 minutes to H³TdR at various times from 1 to 28 hours before fixation, and then recording the frequencies with which individual chromosomes were labelled in cells obtained from such cultures. But because there was a great deal of variation in stage of division among the population of cells used in this experiment, since they were allowed to grow in isotope-free medium both before and after a short period of exposure to H³TdR, one could not identify the precise time of initiation of DNA replication in individual chromosomes. In the present study cultured leucocytes were exposed to H³TdR during the onset and early stages of their DNA synthetic period and then were allowed to terminate their synthesis in isotope-free medium. The onset of DNA synthesis in individual chromosomes, in this case, could be directly identified because any labelled chromosome or chromosome segment must have replicated before the unlabelled chromosomes or parts of chromosomes. The purpose of the present study is, (a) to determine whether or not the onset of replication in the chromosomes of a normal human female complement is asynchronous, as its termination is, (b) if so, whether the initiation of DNA replication in all chromosomes follows a consistent order, and (c) whether such patterns can provide a basis for characterizing individual chromosomes within a group, which are not morphologically identifiable by the present cytological methods.

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Materials and Methods

Leucocytes from peripheral blood from five apparently normal human females (Cases A, B, C, D, and E) were cultured by the standard technique (Moorhead, Nowell, Mellman, Battips, and Hungerford, 1960). It was first determined through another autoradiographic study (A. K. Sinha and B. B. Mukherjee, unpublished data) that DNA synthesis in human cultured leucocytes did not begin until after at least 28 hours of culture. This agreed with the time reported by Lima-de-Faria *et al.* (1961). Tritium-labelled thymidine of low specific activity (H^3TdR , specific activity 0.36 curies/mM, Schwarz BioResearch Inc., N.Y.) was therefore added to cultures from each of the five cases, in a final concentration of $1 \mu\text{Ci/ml.}$, after 22 hours of incubation at 37°C . In some of the cultures from two cases, H^3TdR was added at the time of setting up the cultures. After 42 to 56 hours of incubation each culture was washed three times and the cells were suspended in tracer-free medium, prepared with isologous plasma. Colchicine (5×10^{-5}) was added one hour after the washing. After a total of 70–75 hours, the cells were harvested, treated with hypotonic solution, fixed, flame-dried on slides, and stained with acetic orcein. The slides were then coated with Kodak NTB₃ emulsion, exposed for 7 days, and developed. Metaphase figures underlying the emulsion were restained with Azure B bromide for 7–8 minutes, rinsed in distilled water, and air-dried. Good metaphase figures with overlying grains were photographed and their locations on the slides were recorded. The silver grains were then removed from the autoradiogram by following the technique developed by A. Ross (personal communication). The slides were dipped in a solution of 7.5% potassium ferricyanide for 15 minutes, rinsed thrice with a 24% solution of sodium thiosulphate, and washed in running tap water for 5 minutes. They were then restained with Azure B bromide. Each recorded metaphase figure was re-photographed and this served as a guide in karyotyping the autoradiogram of the same metaphase figure. A series of such double karyotypes from each subject formed the basis of the present study.

The relative positions of the cells, seen at metaphase, in the DNA synthetic period at the time the isotope was removed from the medium were determined by arranging them in a sequence of increasing grain densities. Since H^3TdR was available from the beginning of the synthetic period and was removed from the medium after 42–56 hours of incubation, any labelled chromosome or chromosome segment must have synthesized DNA in the presence of the isotope and any unlabelled chromosome or chromosome segment must have replicated after the removal of the tracer. Furthermore, since DNA synthesis takes place only during the S period, the grain density would be heavier in cells that were closer to the end of the synthetic period at the time the isotope was removed from the culture.

It should be pointed out here that this experiment was based on the assumption that DNA synthesis in cultured leucocytes did not begin without the addition of a mitogenic agent, such as phytohaemagglutinin, in

the culture. The fact that Bond, Cronkite, Fliedner, and Schork (1958) had observed some DNA-synthesizing leucocytes in the circulating blood of normal individuals does not invalidate the present experiment, since the frequency of such cells was found to be only 0.06% or less of the total white cell count. A report by Rubini, Keller, Eisentraut, and Cronkite (1962) should also be mentioned in relation to the present technique. It stated that in human leukaemic blood, as well as in normal dog marrow, the *in vitro* incorporation of H^3TdR into new DNA was very limited, occurring only during the first 20 minutes of the incubation period. The used supernatant containing intact H^3TdR failed to label fresh added cells. But this observation is in complete disagreement with the results obtained by Taylor (1962) in other mammalian cells and also by Bader *et al.* (1963) in cultured leucocytes from normal human females and therefore should not be applied to mammalian cells in general.

Results and Discussion

Metaphase figures with varying grain densities were obtained from cultures of all five cases used in this experiment and a total of 54 karyotypes was prepared and analysed (Table). Although the intensity of labelling varied greatly among metaphase figures from the same individual, in figures with approximately equal grain density corresponding chromosomes or groups of chromosomes usually showed similar labelling patterns. This was true whether such figures came from different cultures of the same subject or from different individuals. Comparable labelling patterns were also observed in figures derived from the cultures in which H^3TdR was added before incubation, which suggests that prolonged exposure to H^3TdR does not necessarily alter the normal control mechanism, at least when the isotope is of low specific activity and used in a very low concentration. These observations demonstrated very clearly a consistency in the time of initiation of DNA replication. It was, therefore, considered appropriate to combine the results of all five cases (Table) in order to determine the presumptive order in which individual chromosomes or groups of chromosomes initiated DNA synthesis.

It can be seen in the Table that the onset of DNA synthesis is not simultaneous in all chromosomes of the human female complements. The grain density of the 54 metaphase figures varied from 30 to 317 grains per figure. The karyotypes containing less than 30 grains were not included in this study in order to avoid any bias arising from failure to distinguish silver grains from plain artefacts (Fig. 1). It can be observed (Table) that both homologues of chromosome 1 and one each of chromosomes 2 and

TABLE*

	Case No.	Grain Counts	Number of Labelled Chromosomes in Each Group								
			1	2	3	4-5	6-12-X	13-15	16-18	19-20	21-22
1	D1	317	2	2	2	4	16	16	6	4	4
2	B1	306	2	2	2	4	16	6	5	4	4
3	B2	229	2	2	2	4	15	6	6	4	3
4	D4	208	2	2	2	4	16	6	6	3	3
5	D2	193	2	2	2	4	15	6	6	4	4
6	D3	164	2	2	2	4	16	5	6	4	4
7	D5	158	2	2	2	3	15-16	5	4	4	4
8	*A1	157	2	2	2	4	16	5	5	4	4
9	B3	147	2	2	2	3	15	5	6	4	3
10	C2	145	2	2	2	4	15	5	5	4	3
11	E4	138	2	2	1	4	15	5	5	3	4
12	*A2	137	2	2	2	4	16	6	5	4	3
13	E3	131	2	2	2	4	14	5	6	3	2
14	*A3	127	2	2	2	4	15	5	6	4	3
15	C3	123	2	2	2	4	15	5	6	4	2
16	C1	119	2	2	1	4	16	6	5	4	2
17	E10	119	2	2	2	2	14	5	5	3	2
18	E6	118	2	2	2	4	14	5	5	1	3
19	*A4	117	2	2	2	3	14	6	6	4	3
20	E1	114	2	2	2	4	15	6	6	4	2
21	*C4	113	2	2	2	4	15	4	4	3	2
22	D6	113	2	2	2	4	15	6	6	3	2
23	E2	113	2	2	2	4	15	6	5	4	3
24	*A5	107	2	2	I-2	4	15	5	5	3	1
25	*C5	104	2	2	I	3	12	5	5	3	2
26	D7	102	2	2	2	4	15	5	4	3	3
27	E5	101	2	2	2	4	14	5	5	2	3
28	E7	99	2	2	2	4	14	5	3	2	3
29	E11	97	2	2	1	4	12	4	4	3	1
30	D8	90	2	2	2	4	13	4	5	2	2
31	E8	89	2	2	2	3	14	4	5	1	3
32	B4	87	2	2	2	3	14	3	3	3	2
33	E9	83	2	2	2	3	13	5	5	3	I-2
34	*A6	80	2	2	2	2	12	3	5	4	2
35	*A7	77	2	2	2	2	13	4	3	2	1
36	B5	73	2	2	2	4	12	3	5	3	1
37	*A8	67	2	2	1	3	13	3	4	1	1
38	C6	60	2	2	2	2	9	4	4	3	1
39	B6	59	2	2	2	3	10	3	4	2	1
40	*C7	59	2	2	2	3	11	4	3	1	1
41	E17	57	2	2	1	3	9	3	3	2	2
42	D9	54	2	2	2	3	10	4	4	2	0
43	E12	53	2	2	1	3	10	2	4	2	2
44	E14	52	2	2	2	2	11	3	3	2	2
45	E15	50	2	1	2	3	11	4	2	1	2
46	E16	49	2	2	2	3	11	3	2	0	1
47	*C8	47	2	2	0	2	11	1	3-4	1	1
48	D11	46	2	1	2	2	5	4	2	1	1
49	D10	41	1	2	2	2	9	4	4	1	1
50	*C9	40	2	1	2	2	8	2	3	1	0
51	E18	39	2	2	1	2	9	2	3	1	0
52	C10	38	2	1	1	2	7	2	4	I-2	0
53	E19	36	2	1	1	3	7	2	4	1	0
54	*C11	30	2	1	1	2	7	2	1-2	1	0

* The relative positions of all the 54 metaphase figures in the DNA synthetic period at the time the isotope was removed from the medium were determined by arranging them in a sequence of increasing grain densities. The cells with comparatively more grains, such as metaphase number 1 with 317 grains, are closer to the end of the synthetic period than those with fewer grains, such as number 54 which contains only 30 grains. A chromosome that shows one or more overlying silver grains is considered labelled. The metaphase figures marked with an asterisk were taken from the cultures in which H^3TdR was added before incubation.

3 were labelled in a metaphase figure containing as low as 30 grains, so that they must have begun replication of their DNA relatively early, but the second homologues of chromosomes 2 and 3 did not show any labelling until the grain density reached approximately 39 to 40 grains. Only two of the four chromosomes in group 4-5 were found to have started replication in seven out of eight metaphase figures containing 30 to 47 grains, but in a majority of the cells which had continued their DNA synthesis a little further in the presence of the isotope, three chromosomes of this group were

found to be labelled. In 25 out of 30 karyotypes which showed more than 89 grains, all four chromosomes of this group had started replication. Five to nine chromosomes in group 6-12+X were labelled in cells containing approximately 30 to 46 grains but as the grain density went higher the number of labelled chromosomes increased gradually, and by the time the grain count per metaphase figure reached approximately 87 grains, 13 to 14 chromosomes appeared to have started replication. One or more of the 16 chromosomes of this group remained unlabelled in cells with a grain count of 145 or lower.

FIG. 1. A very lightly labelled metaphase figure. Each mitotic figure has been photographed before and after the application of the photographic emulsion. Karyotype has been prepared from the matched photographs.

The number of chromosomes that had initiated DNA synthesis in group 13-15 increased gradually from 2 to 5 in metaphase figures with grain counts ranging from 30 to 99 grains, and in 16 out of 23 cells containing 99 to 164 grains only five chromosomes of this group were found to be labelled. One of the four chromosomes in group 19-20 began replication considerably earlier than the remaining three, and in eight or nine out of ten figures which showed 30 to 50 grains only one chromosome appeared to have started replication. A little later in the DNA synthetic period another chromosome started synthesis, but one chromosome of the group remained unlabelled until the grain counts per metaphase figure reached approximately 113 or higher. In the karyotype which showed less than 40 grains, all four chromosomes in group 21-22 were unlabelled, and in 10 out of 15 metaphase figures containing 40 to 77 grains only one chromosome was found to be labelled. All four chromosomes of this group had begun synthesis only when the labelling intensity of metaphase figures had reached approximately 138 grains or higher. One chromosome each in groups 6-12+X, 13-15, 16-18, and 21-22 appeared to be the last chromosome in the whole complement to begin replication and in many metaphase figures containing approximately 137 grains or higher these chromosomes still remained unlabelled. As a group, group 21-22 was the last to initiate replication (Table).

In general, a relation was found to exist between the size of a chromosome and its time of initiating DNA synthesis (Fig. 2). This relation varied

FIG. 2. Regression of percentage of chromosomes labelled on relative length at various grain densities.

considerably with the grain density of the metaphase figures. In cells with very low grain counts (less than 30 grains) only a few chromosomes were labelled, while in figures with very high grain density (200 grains or higher) nearly all chromosomes were found to have started replication. Metaphase figures from three cases were arbitrarily divided into three convenient groups according to their grain densities. Mean values were obtained for each case, so that the regressions were based on three means for each relative length. Significant regressions were obtained for each of the three levels of grain density. The magnitude of the regression coefficient declined with increasing labelling intensity.

Although in general a relation did exist between the size and the time of initiation of DNA synthesis of a chromosome, there were exceptions to this general pattern. All chromosomes that belonged to a particular size group did not start synthesis simultaneously, and it was quite obvious from the table that some of the smaller chromosomes began replication before some of the larger chromosomes. For example, one of the four chromosomes in group 21-22 started replication in a majority of the metaphase figures containing 41 to 77 grains, while one or two chromosomes in group 19-20, two or three chromosomes in each of groups 16-18 and 13-15, and three or four chromosomes in group 6-12+X, still remained unlabelled. Similarly, all four chromosomes in group 19-20 were found to have started replication in 80% of the metaphase figures containing 113 grains and higher, while all of the chromosomes in groups 16-18, 13-15, and 6-12+X did not begin synthesis in a majority of the metaphases until their grain density reached approximately 145 or higher. Even homologues of chromosomes 2 and 3 did not appear to initiate synthesis simultaneously despite their similar sizes, and such asynchronous initiation of DNA replication among homologous chromosomes was further indicated by the fact that only one out of four chromosomes in each of groups 19-20 and 21-22 started replication considerably earlier than the rest. It is interesting to note also that at certain stages during the S period there appears to be a recognizable time interval before the next chromosome within a group starts replication. For example, in group 4-5 (Table and Fig. 3A) two of the four chromosomes began replication earlier than the other two and they continued replication for some time before the third chromosome started. A little later during the synthetic period the fourth chromosome initiated DNA synthesis. Such an interval exists also between the first and second

FIG. 3A. Autoradiograms of 10 sets of chromosomes in group 4-5 arranged in a sequence of increasing grain densities of the metaphase figures from which they are taken. In a majority of the metaphase figures containing approximately 47 grains or less only two chromosomes have commenced replication; a little later in the S period another chromosome begins replication, and in most of the cells with higher than 89 grains all four chromosomes in this group have started replication.

FIG. 3B. Four homologous pairs of each of chromosomes 1 and 2 taken from metaphase figures with different grain densities. In lightly labelled cells the beginning of DNA synthesis appears to be restricted to a single site in many of the chromosomes, but at a slightly later stage during the S period more than one site is seen to be involved in simultaneous replication.

chromosomes of each of the groups 19-20 and 21-22 (Table and Fig. 4A and 4B). In another stage during the synthetic period, however, two or more chromosomes within the same morphological group may have the same initiation time, or the interval between their time of initiation of DNA replication is so short that anything from one to all of them could be labelled in cells containing the same grain densities. For example, in a majority of metaphase figures, with the labelling intensities ranging from 80 to 137 grains, either two or three of the four chromosomes in group 21-22 were found to have started replication, which indicates that these chromosomes begin replication almost simultaneously.

It has been clearly observed (Table) that a recognizable interval exists between the time of initiation of the second, third, and fourth chromosomes in group 4-5, and between the first and second chromosomes in each of groups 19-20 and 21-22. Similarly, one chromosome each in groups 6-12 + X, 13-15, 16-18, and 21-22 starts replication later

than all other chromosomes in the complement. Assuming that the time of initiation of DNA synthesis for each chromosome is constant in every cell, such a pattern may provide some basis for identifying one or more individual chromosomes within different morphological groups.

The beginning of DNA synthesis appears to be restricted to a single site in many of the human chromosomes (Fig. 3B), but the discontinuous and differential intensity of labelling along individual chromosomes, at a slightly later stage, during the S period, suggests strongly the involvement of multiple sites in simultaneous replication rather than a linear progression. The presence of silver grains along the entire length of the late-replicating human X chromosome after only 10 minutes of pulse treatment (German, 1962) also supports this idea. The sedimentation polydispersity of denatured phage DNA after analytical ultracentrifugation has been interpreted by Davison, Freifelder, and Holloway (1964) as an indication for the existence

FIG. 4A. Autoradiograms of 16 sets of chromosomes in group 19-20 arranged in a sequence of increasing grain densities of the metaphase figures from which they are taken. In metaphase figures containing approximately 50 grains or less only one chromosome in this group has started replication. In comparatively heavily labelled cells (113 grains and higher) all four chromosomes are replicating.

FIG. 4B. Chromosomes of group 21-22 from eight different metaphase figures arranged in the same way as the chromosomes in group 19-20. One of the four chromosomes in this group starts replication when the grain density of a metaphase figure reaches approximately 40 grains or higher. This is followed by two other chromosomes which begin replication almost simultaneously. The fourth chromosome initiates DNA synthesis later than the other three.

of 'single-strand interruptions' or weak points within viral DNA at which polynucleotides rupture during denaturation. In his study on the replicative patterns of *Drosophila* salivary gland chromosomes, Plaut (1963) observed multiple simultaneously replicating points along their lengths and suggested that these synthesizing sites probably represented the location of 'molecular ends'. If these interpretations are correct, it is reasonable to infer that at least the longer human chromosomes contain multiple 'molecular ends' along their DNA double helices, which are capable of initiating DNA synthesis independently and simultaneously.

It has been observed that homologues of many autosomes, and the X chromosomes in the human complement (Bader *et al.*, 1963; Lima-de-Faria *et al.*, 1961) as well as in other mammalian complements (Mukherjee and Sinha, 1963, 1964), differ in their temporal and linear sequence of DNA replication. If chromosomal DNA synthesis starts at the 'molecular ends' and proceeds sequentially in a semiconservative fashion, and if specific 'molecular ends' follow a definite temporal sequence in initiating DNA synthesis, the observed homologue asynchrony would imply a differential distribution of 'molecular ends' between homologues.

Summary

The pattern of initiation of DNA replication in chromosomes of five normal human females was studied by exposing the cultured leucocytes to H^3TdR at the beginning of their DNA synthetic period and then removing the isotope from the medium after 42–56 hours of incubation. It has been observed that the onset of DNA synthesis is not simultaneous in all chromosomes of the human female complement. In metaphase figures which contained 30 to 38 grains, both homologues of chromosome 1, one each of chromosomes 2 and 3, two chromosomes in group 4–5, five to nine chromosomes in group 6–12+X, two chromosomes in group 13–15, three to four chromosomes in group 16–18, only one chromosome in group 19–20, and none of the chromosomes in group 21–22 were labelled. One chromosome each in groups 6–12+X, 13–15, 16–18, and 21–22 appeared to be the last chromosomes in the whole complement to begin replication. At certain stages during the S period there exists a recognizable time interval between the time of initiation of DNA replication of two chromosomes of the same morphological group, which may provide some basis for identifying a number of individual chromosomes. Homologue asynchrony has been found to exist in several chromosomes. As a group, group 21–22 was the last to begin replication. In general, correlation was found to exist between the size of a chromosome and its time of initiation of DNA replication, but there were exceptions.

Discontinuous and differential intensity of labelling along many chromosomes during the synthetic period suggests the involvement of multiple sites in simultaneous replication, and these synthesizing sites may represent the location of 'molecular ends' along DNA double helices. If chromosomal DNA synthesis starts at the 'molecular ends' and specific 'ends' follow definite temporal sequences, their variable number or differential distribution along homologous or non-homologous chromosomes may contribute to asynchronous initiation of DNA replication.

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