CHROMOSOMAL DNA SYNTHESIS IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Analysis of labeling patterns in three chromosome segments of *Drosophila melanogaster* has shown that the replicative activity within chromosomes is temporally ordered. Moreover, specific labeling patterns on one chromosome occur with specific patterns on another chromosome with a very high degree of correlation. This circumstance leads to the conclusion that DNA synthesis among all the regions in the three chromosome segments studied is coordinated. The various labeling patterns observed in any one chromosome and the combinations of labeling patterns observed in all three chromosome segments can be arranged in ordered arrays, if one assumes that the DNA synthesis in each chromosome region will go to completion without stopping once it has started. Such arrays can serve as models for the temporal order of DNA synthesis among chromosome regions. They predict that in any one chromosome DNA replication begins and ends at very few loci and that synthesis at a larger number of points occurs at an intermediate time.

INTRODUCTION

Recent work on chromosomal DNA synthesis has indicated that the process is subject to temporal control. Not only is the bulk of DNA synthesis, that synthesis usually assumed to be directly involved in chromosome duplication, restricted to a particular portion of the interphase period (Howard and Pelc, 1951), but some chromosomes or chromosome segments regularly show DNA synthesis during specific portions of the over-all synthetic period (Lima-de-Faria, 1961; Painter, 1961; Taylor, 1960). In mitotically dividing cells in various organisms chromosomes have been recognized as replicating either early or late during the S period (Hsu et al., 1964), and a preliminary correlation has been established between heterochromatin, late DNA synthesis, and transcriptive inactivity (Taylor, 1960; Lyon, 1961; Brown, 1966). While the generality of this correlation remains to be critically tested, it appears likely that the timing of the replication of specific segments of chromosomal DNA may have implications for the expression of genetic information as well as for the structural duplication of the chromosome.

The radioautographic study of asynchronous chromosomal DNA synthesis can be carried out with greater precision in polytene chromosomes than in mitotic chromosomes which can be examined only in a contracted division state. Tritiated thymidine pulse-labeling of dipteran salivary glands by a number of investigators (Plaut, 1963; Keyl and Pelling, 1963; Gay, 1964; Gabrusewycz-Garcia, 1964; Ritossa, 1964; Swift, 1964; Fujita, 1965; Pavan, 1965; Berendes, 1966; Plaut et al., 1966; Nash and Bell, 1968; Rodman, 1968) has shown two distinct patterns of chromosomal DNA synthesis. Either chromosomes exhibit thymidine incorporation along their entire length, or synthesis of

DNA is restricted to isolated points. Both of these patterns as well as intermediates, where the distribution and extent of labeled segments approaches continuous labeling along the chromosome, can be seen among the nuclei in a single pulse-labeled salivary gland. It is generally assumed that all of these patterns of DNA synthesis represent phases in the normal duplication of chromosomal DNA. The detailed examination of such patterns within individual polytene chromosomes has led to the conclusion that the DNA of each chromosome can be thought of as being subdivided into a large series of replicating entities whose synthetic activity is coordinated in time (Plaut et al., 1966). The present paper extends the concept of ordered replication from within to between chromosomes and presents a model for the over-all sequence of chromosomal DNA replication in *Drosophila melanogaster*.

MATERIALS AND METHODS

Fly Culture

The flies used in all experiments were Oregon-R wild type *Drosophila melanogaster*. The culture medium contained molasses, cornmeal, brewer's yeast, and propionic acid. Well-fed, 1 wk-old adult flies (grown at room temperature) were placed on fresh culture medium for 1 hr, usually in the early afternoon. During this period 50–100 eggs were laid. The adult flies were then removed from the culture bottles which were placed in an 18°C incubator. (Larvae were grown at 18°C to insure production of large salivary glands. Adult flies were reared at room temperature for maximum mating and egg laying activity.) Egg hatching occurred within 48 ± 4 hr at 18°C. However, third instar larvae in these cultures formed puparia during a 24–36 hr period. Chronologically, all larvae used in these experiments were on the average 290 ± 30 hr from oviposition and 242 ± 4 hr from hatching. Developmentally they were in late third instar. All were among the first group of larvae to cease feeding and to crawl out of the medium onto the walls of the culture bottle. Attempts to increase developmental synchrony among third instar larvae through modification of the above culture method were unsuccessful.

Chromosome Labeling Experiments

LABELING OF CHROMOSOMES; PREPARATION AND STAINING OF SLIDES: Salivary glands were excised from late third instar larvae in modified Ringer's solution (Ephrussi and Beadle, 1936). The glands were incubated for 17 ± 2 min in Ringer's solution which contained tritiated thymidine (New England Nuclear Corporation, Boston, Mass; specific activity, 10.6 c/mmole; Schwarz BioResearch Inc., Orangeburg, N.Y; specific activity, 14.0 c/mmole) at a concentration of 10 μc/ml. Following this incubation the glands were squashed on gelatinized slides in a drop of 45% acetic acid under a No. 2 cover slip. Cover slips were removed after liquid nitrogen freezing. The tissue was postfixed on the slides in absolute ethanol-acetic acid (3:1) and then hydrated through a graded series of aqueous ethanol solutions. After they had been thoroughly rinsed in deionized water, the slides were stained with aceto-orcein, and cover slips were mounted with Euparal according to the method described by Plaut et al. (1966).

SELECTION OF NUCLEI FOR ANALYSIS: The stained squash preparations were carefully examined with the phase contrast microscope, and the location on each slide of every analyzable nucleus was recorded. A nucleus was selected for analysis if it contained, in a well-squashed condition, at least two of the following three chromosome segments: the terminal portion of the right arm of chromosome II, the terminal portion of the left arm of chromosome III, and the terminal portion of the X-chromosome. These segments were chosen for analysis because of the ease with which they can be identified in squash preparations. Only terminal portions of each chromosome arm were observed (approximately 15% of the total length of each arm) because of the low frequency of squash preparations in which the entire length of even one chromosome arm is suitably preserved. Observation of short segments also simplified the labeling patterns which had to be analyzed. Fig. 1 is a schematic representation of the terminal portions of II R, III L, and the X, which were studied. The chromosome segments have been subdivided into a series of numbered and lettered regions. Each horizontal row of letters or numbers corresponds to a different series of subdivisions of the chromosomes. The two horizontal rows which are marked with an asterisk show the cytological regions defined by Bridges (1938). The horizontal row of numbered segments which is marked with a double dagger defines the cytological regions observed in the present study. Each of these latter regions designates a portion of the chromosome whose individual DNA synthetic activity can be resolved radioautographically from that in adjacent regions.

PHOTOGRAPHY: Every analyzable nucleus was photographed with phase optics on Kodak high contrast copy panchromatic film before radioautographic emulsion was applied in order to obtain a clear record of the banding pattern in each chromosome. This aided the precise localization of radioautographic grains above specific chromosome regions.

RADIOAUTOGRAPHY: After the slides were pho-

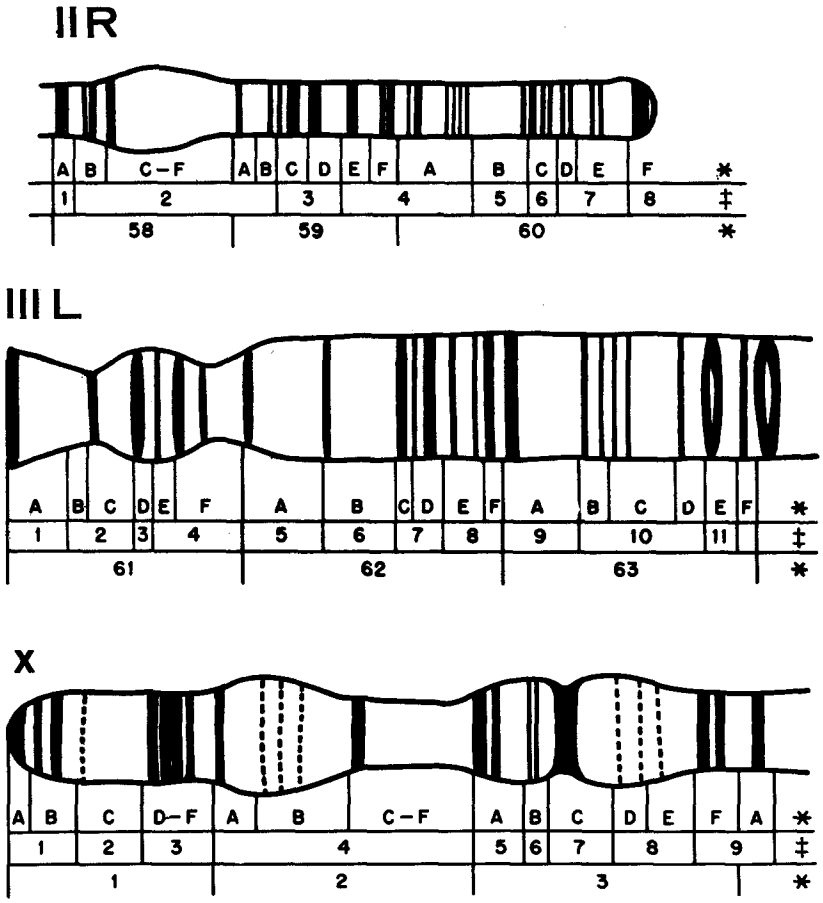

FIGURE 1 Terminal segments of chromosomes II R, III L, and X. The chromosomes have been subdivided into a series of numbered and lettered regions. The upper and lower rows of subdivisions (asterisks) in each chromosome segment show the cytological regions defined by Bridges (1938). The middle row of numbered divisions (double dagger) shows the regions observed in the present study.

tographed, they were placed in absolute ethanol overnight to remove the cover slips and mounting medium. The slides were then hydrated in a graded series of aqueous ethanol solutions. Following a wash in deionized water, radioautographic stripping film (Kodak AR-10) was applied in the dark room by using a water bath held at 25°C. The slides were placed in light-tight boxes and stored at 2°–4°C for 14–21 days. They were then developed in Kodak D-19b developer at 18°C.

ANALYSIS OF RADIOAUTOGRAPHS: All of the nuclei which had been photographed before the radioautographic stripping film was applied were relocated with the phase-contrast microscope. In a total of 11 separate labeling experiments an average of 43 ± 3.8% of these nuclei contained pertinent chromosome segments which were labeled. A particular chromosome segment was considered labeled if the grain density in the cluster of silver grains in the emulsion above this segment was at least twice the average grain density above equivalent areas on the same slide which contained no tissue (background grains). This usually meant that a tight cluster of at least four silver grains had to be seen above a chromosome region before it was considered labeled. Those chromosome segments which were covered *in toto* by silver grains in radioautographs were said to be continuously labeled. Fig. 2 shows a nucleus where all chromosomes are continuously labeled. Chromosome segments where only some regions were labeled (Fig. 3 *b*) were said to be discontinuously or spot labeled. This terminology differs somewhat from that used by Plaut (1963) who used the term "continuously labeled" to refer to a polytene nucleus where all regions in all chromosomes were labeled. In this study, a chromosome segment is referred to as con-

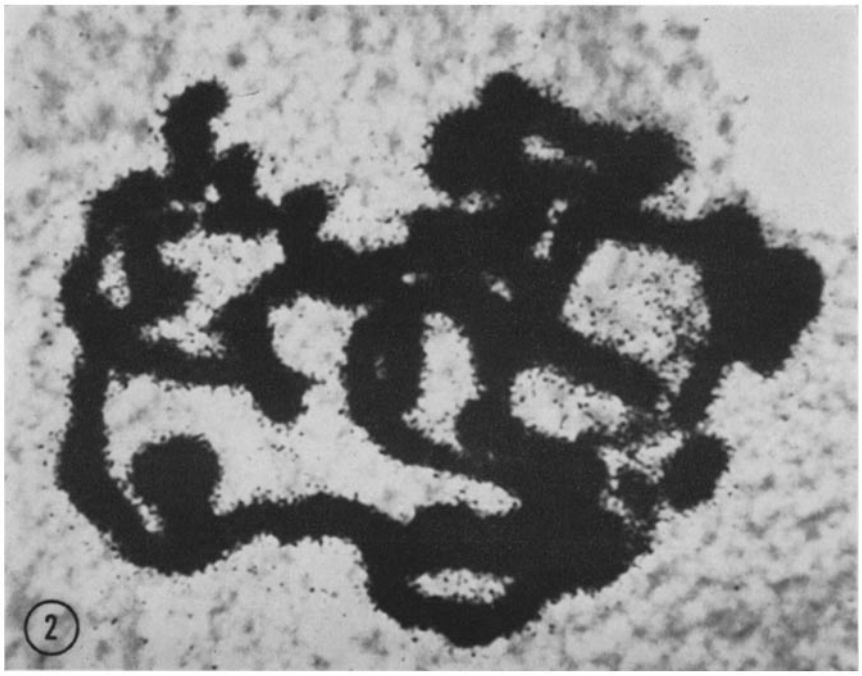

FIGURE 2 Salivary gland polytene chromosomes continuously labeled with tritiated thymidine. The entire chromosome complement from a single nucleus is shown here. Time of incubation in isotope was 15 min. Film exposure time was 2 wk at 4°C. Magnification about 1200.

tinuously labeled even though other chromosomes or chromosome regions in the same nucleus may be spot labeled.

The various combinations of labeled regions seen in the chromosome segments are referred to as labeling patterns. For example, an X chromosome terminal segment (Fig. 1) labeled in regions 1 and 7 is said to exhibit labeling pattern (1, 7). An unlabeled X-chromosome segment is said to exhibit labeling pattern (−), and a continuously labeled X segment exhibits labeling pattern (1, 2, 3, 4, 5, 6, 7, 8, 9). Fig. 3*b* shows a nucleus where the X-chromosome segment has labeling pattern (1, 7) and the II R segment labeling pattern (1, 3, 8).

Analysis of the radioautographs involved the keeping of a careful record of the labeling patterns observed in each nucleus. In addition, the presence or absence of labeling in the chromocenter was noted. The data from 11 separate experiments were pooled. Approximately 15% of all labeled nuclei were discarded because of uncertainty as to whether or not a region in a chromosome segment was indeed labeled.

ENZYME CONTROLS: The specificity of tritiated thymidine as a label for DNA was tested with enzymes. These tests had to be performed on unstained squash preparations since DNase was inactive when used on preparations which had been stained with aceto-orcein. DNase solutions (Worthington Biochemical Corp., Freehold, N. J.) contained 0.3 mg of DNase per milliliter of aqueous 0.004 M $MgSO_4$. The pH of the DNase solution was adjusted to 7.0 with 0.1 N NaOH. RNase solutions (Worthington) contained 0.3 mg of RNase per milliliter of deionized water. The pH was adjusted to 7.0 with 0.1 M Na_2HPO_4. Slides with unstained, tritiated thymidine-labeled salivary gland squashes which had otherwise been treated exactly as stained preparations were exposed to either DNase or RNase solution for 3½ hr at 37°C (five slides per treatment; approximately 100 nuclei per slide). This was done just before application of the radioautographic stripping film. In the DNase-treated tissue no significant labeling was seen in the emulsion above chromosomes in any nuclei. In the RNase-treated tissue chromosomes were labeled as heavily as were chromosomes in untreated controls. It was concluded that the label observed in our preparations resulted from incorporation of tritiated thymidine specifically into DNA.

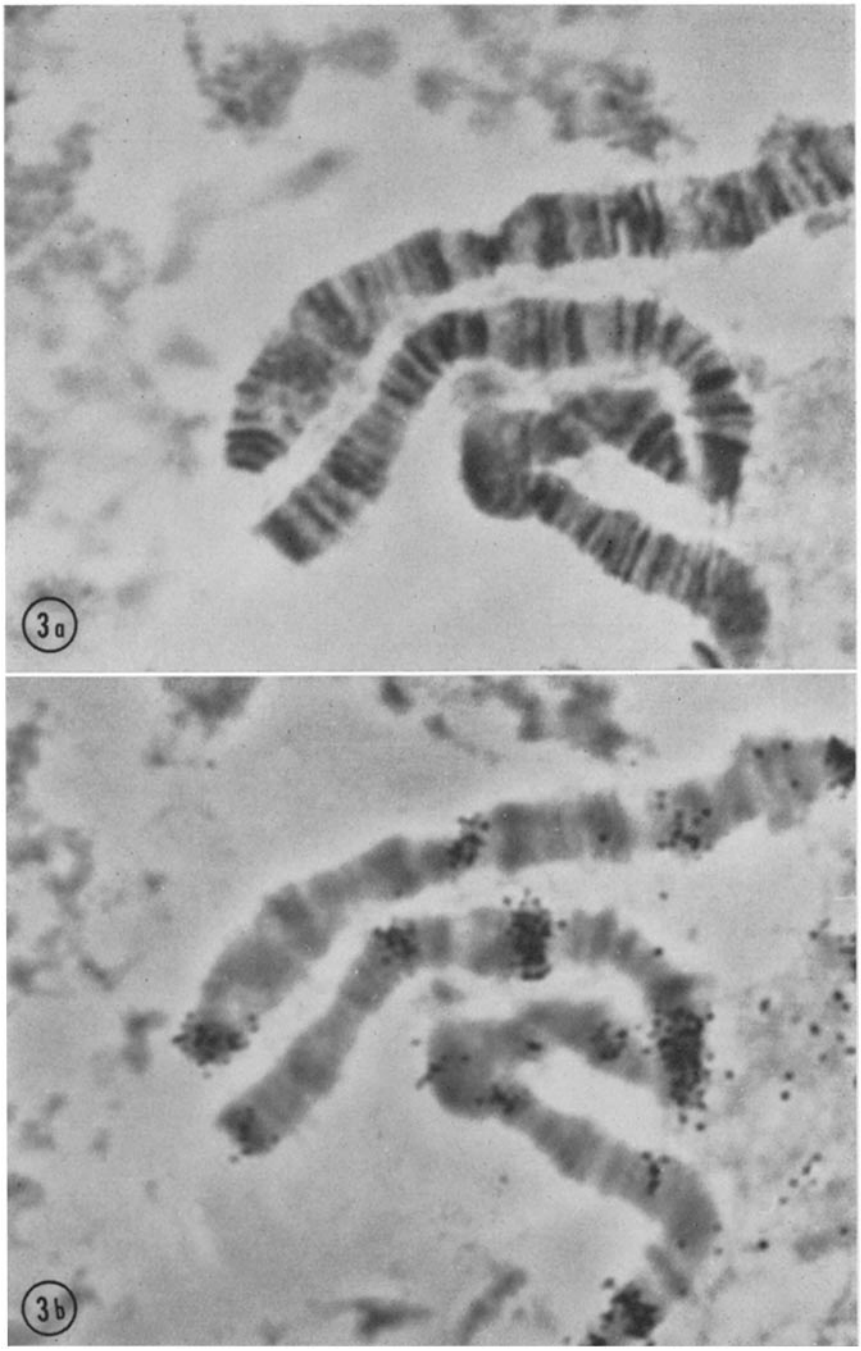

FIGURE 3 *a* Chromosome segments X (the upper chromosome) and II R before application of radioautographic film. Magnification about 2200.

FIGURE 3 *b* Chromosome segments X and II R discontinuously labeled with tritiated thymidine. The X segment (the upper chromosome) exhibits labeling pattern (1, 7). The II R segment exhibits labeling pattern (1, 3, 8). Labeled nucleolar material can be seen at the right border of the photograph. Time of incubation in isotope was 15 min. Film exposure time was 2 wk at 4°C. Magnification about 2200.

TABLE I

Tritiated Thymidine Labeling in the II R-Chromosome Segment

	II R Segment							
Region	1	2	3	4	5	6	7	8
Chromosomes labeled in this region	397	59	347	59	51	82	59	175
Absolute labeling frequency	0.407	0.061	0.356	0.061	0.052	0.084	0.061	0.179

Labeling pattern	No. observed	Absolute frequency
(–)	578	0.593
(1)	50	0.051
(1, 3)	172	0.176
(1, 3, 8)	93	0.095
(1, 3, 6, 8)	23	0.024
(1, 2, 3, 4, 6, 7, 8)	8	0.008
(1, 2, 3, 4, 5, 6, 7, 8)	51	0.052
	975	0.999

The upper portion of the table gives the labeling data for individual regions in the II R segment. The number of times each of the eight regions was found labeled in 975 II R segments and the absolute frequency this number represents are indicated. The labeling patterns which were observed in the same 975 II R segments are shown in the lower portion of the table. The number of times each pattern was observed and the absolute frequency of each pattern are given.

RESULTS

DNA Synthesis in Individual Chromosome Segments

Table I shows the frequency with which each of the eight regions in the II R chromosome segment was found labeled following a pulse of tritiated thymidine. A total of 975 II R segments was observed. It can be seen that each region labels with a characteristic frequency which is different from that for other regions. Also listed in Table I are the seven different combinations of labeled regions (labeling patterns) which were seen in this same sample of 975 II R segments. The observed frequency of each pattern is given. Table II shows the labeling frequency for each of the 11 regions in 760 III L segments and lists the 17 different labeling patterns which were observed in this chromosome. Table III presents the labeling data for 535 X-chromosome segments. 13 different labeling patterns were observed in the X.

Attention is directed to two aspects of the data presented in Tables I–III which indicate that DNA synthesis among the regions in each individual chromosome segment is coordinated.

(*a*) There are fewer labeling patterns than would be predicted by chance; if chance alone determines which chromosome regions are found labeled in single nuclei following a pulse of tritiated thymidine (i.e., if DNA synthesis in each region is an independent event), then 2^n different labeling patterns should be observed in a large sample of chromosomes with n different regions. However, only seven of a possible $2^8 = 256$ labeling patterns were observed in 975 II R-chromosome segments. Similarly, only 17 of a possible $2^{11} = 2048$, and 13 of a possible $2^9 = 512$ patterns were seen in 760 III L and 535 X segments, respectively. This indicates that DNA synthesis does not occur at random among the regions in individual chromosome segments.

(*b*) The observed frequency of each labeling pattern is significantly different from that predicted by chance; the labeling frequency of each region in a chromosome segment (Tables I–III) can be thought of as the probability of finding that region labeled in a nucleus regardless of what other regions are labeled. If replication in each region is an independent event, then the probability of observing labeling in any given combination

TABLE II

Tritiated Thymidine Labeling in the III L-Chromosome Segment

	III L Segment										
Region	1	2	3	4	5	6	7	8	9	10	11
Chromosomes labeled in this region	118	80	61	79	82	67	100	80	148	49	106
Absolute labeling frequency	0.155	0.105	0.080	0.104	0.108	0.088	0.132	0.105	0.195	0.064	0.139

Labeling pattern	No. observed	Absolute frequency
(–)	583	0.767
(7)	1	0.001
(9)	42	0.055
(1)	28	0.037
(9, 11)	4	0.005
(7, 9, 11)	9	0.012
(1, 9, 11)	3	0.004
(1, 7, 9, 11)	7	0.009
(4, 7, 8, 9, 11)	1	0.001
(1, 5, 7, 9, 11)	2	0.003
(1, 2, 5, 7, 9, 11)	1	0.001
(1, 2, 5, 7, 8, 9, 11)	1	0.001
(2, 4, 5, 7, 8, 9, 11)	2	0.003
(1, 2, 4, 5, 7, 8, 9, 11)	9	0.012
(1, 2, 4, 5, 6, 7, 8, 9, 11)	6	0.008
(1, 2, 3, 4, 5, 6, 7, 8, 9, 11)	12	0.016
(1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11)	49	0.064
	760	0.999

of regions (regardless of what other regions are labeled) should be equal to the product of the labeling frequencies for each region. For example, in the II R segment, the labeling frequencies for regions 1, 3, and 8 are 0.407, 0.356, and 0.179, respectively. If DNA synthesis in each of these regions were an independent event, the probability of observing simultaneous labeling in regions 1, 3, and 8 (regardless of what other regions are labeled) should be the product (0.407) (0.356) (0.179) = 0.026. This frequency, then, is the probability of observing *all* labeling patterns which contain label in regions 1, 3, and 8, i.e. patterns (1, 3, 6, 8), (1, 2, 3, 4, 5, 6, 7, 8), etc. It includes the expected chance frequency of finding label *only* in regions 1, 3, and 8, producing labeling pattern (1, 3, 8). Thus, the frequency 0.026 is the upper limit to the probability of observing labeling pattern (1, 3, 8) by chance. It can be shown in a maximum likelihood test (Howard, 1967) that the observed frequency of pattern (1, 3, 8) (0.095, Table I) is significantly different from 0.026. It can be shown with the same statistical test that the observed frequencies of all the labeling patterns in each of the three chromosome segments differ significantly from the maximum likelihood probabilities predicted by chance. It is concluded from this that DNA synthesis among the regions in each of the chromosome segments analyzed is coordinated; individual labeling patterns are not produced by the random association of replicating regions in single nuclei.

DNA Synthesis in Three Chromosome Segments

Table IV lists the combinations of labeling patterns seen in single nuclei in chromosome segments II R, III L, and X. Each horizontal line represents an observed triplet of labeling patterns.

TABLE III

Tritiated Thymidine Labeling in the X-Chromosome Segment

	X Segment								
Region	1	2	3	4	5	6	7	8	9
Chromosomes labeled in this region	166	55	84	51	90	75	198	81	152
Absolute labeling frequency	0.310	0.103	0.157	0.095	0.168	0.140	0.370	0.151	0.284

Labeling patterns	No. observed	Absolute frequency
(–)	326	0.609
(7)	43	0.080
(1)	5	0.009
(1, 7)	9	0.017
(1, 9)	6	0.011
(1, 7, 9)	56	0.105
(1, 5, 7, 9)	5	0.009
(1, 3, 5, 7, 9)	4	0.007
(1, 5, 7, 8, 9)	1	0.002
(1, 3, 5, 7, 8, 9)	5	0.009
(1, 3, 5, 6, 7, 8, 9)	20	0.037
(1, 2, 3, 5, 6, 7, 8, 9)	4	0.007
(1, 2, 3, 4, 5, 6, 7, 8, 9)	51	0.095
	535	0.997

The frequency with which this combination of patterns was observed is indicated at the right end of each line. For example, 96 of a total of 197 nuclei were observed in which all three chromosome segments were unlabeled. 12 nuclei were observed in which the II R segment exhibited labeling pattern (1, 3), the III L segment was unlabeled (–), and the X segment was labeled in region 7. It has been shown (Howard, 1967) that the observed frequency of this last combination of labeling patterns is significantly different from the value predicted by chance. This also holds true for all of the other observed combinations of labeling patterns seen in the three chromosome segments. This suggests that the combinations of labeling patterns on different chromosomes are related in time, and this leads to the conclusion that DNA synthesis among the regions in different chromosomes as well as within individual chromosomes is coordinated.

DISCUSSION

Sequence Analysis of Labeling Patterns

Having established that DNA synthesis among chromosome regions is coordinated, one would like to know the actual sequence in which the various regions are replicated. Information about sequence should be obtainable from analysis of all the labeling patterns which are seen in a chromosome. If each labeling pattern is a stage in a cycle of chromosome duplication, then there must be an ordered sequence of such stages (patterns) which represents the over-all temporal sequence of DNA synthesis in the chromosome. Of course, one can never be certain that an array of labeling patterns, built on arbitrary assumptions, is in fact the correct sequence. However, if a collection of labeling patterns can be easily arranged in sequence using a minimum of simple assumptions, then it is not unreasonable to postulate that such an array bears some relationship to the actual sequence of chromosomal DNA synthesis and can serve as a useful model for further experimental testing.

Table V shows that all 13 labeling patterns observed in the X-chromosome segment can be arranged in sequence. In order to do this it is necessary to assume that DNA synthesis in each chromosome region is uninterrupted, i.e. that DNA synthesis once initiated in a chromosome region goes to completion without stopping during

TABLE IV

Triplets of Labeling Pattern Seen in Single Nuclei in Chromosome Segments II R, III L, and X

II R	III L	X	No. observed	Absolute frequency
(–)	(–)	(–)	96	0.487
(1)	(–)	(–)	6	0.030
(1)	(–)	(7)	3	0.015
(1, 3)	(–)	(–)	7	0.036
(1, 3)	(–)	(7)	12	0.061
(1, 3)	(–)	(1)	1	0.005
(1, 3)	(–)	(1, 9)	3	0.015
(1, 3)	(–)	(1, 7, 9)	3	0.015
(1, 3)	(9)	(1, 7)	1	0.005
(1, 3)	(9)	(1, 7, 9)	2	0.010
(1, 3)	(1)	(1, 7, 9)	2	0.010
(1, 3, 8)	(–)	(7)	1	0.005
(1, 3, 8)	(9)	(1, 7, 9)	1	0.005
(1, 3, 8)	(1)	(1, 7, 9)	2	0.010
(1, 3, 8)	(7, 9, 11)	(1, 7, 9)	1	0.005
(1, 3, 8)	(1, 7, 9, 11)	(1, 5, 7, 9)	1	0.005
(1, 3, 8)	(1, 5, 7, 9, 11)	(1, 3, 5, 7, 8, 9)	1	0.005
(1, 3, 6, 8)	(1, 2, 3, 4, 5, 6, 7, 8, 9, 11)	(1, 3, 5, 6, 7, 8, 9)	3	0.015
(1, 2, 3, 4, 6, 7, 8)	(1, 2, 4, 5, 6, 7, 8, 9, 11)	(1, 3, 5, 7, 8, 9)	1	0.005
(1, 2, 3, 4, 6, 7, 8)	(1, 2, 4, 5, 6, 7, 8, 9, 11)	(1, 3, 5, 6, 7, 8, 9)	1	0.005
(1, 2, 3, 4, 5, 6, 7, 8)	(1, 2, 3, 4, 5, 6, 7, 8, 9, 11)	(1, 3, 5, 6, 7, 8, 9)	1	0.005
(1, 2, 3, 4, 5, 6, 7, 8)	(1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11)	(1, 3, 5, 6, 7, 8, 9)	1	0.005
(1, 2, 3, 4, 5, 6, 7, 8)	(1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11)	(1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12)	47	0.238
			197	0.997

The frequency with which each three-way combination of patterns was observed is indicated at the right end of each horizontal line.

one cycle of chromosome duplication. No further assumptions are necessary when ordering the various patterns. In particular, no assumptions are made about the position of any particular labeling pattern in the array. In Table V the labeling patterns have been placed one above the other. Each horizontal line represents an X-chromosome with a different labeling pattern. Each number on a horizontal line represents the occurrence of DNA synthesis in the chromosome region which bears that number (i.e., the appearance of a spot of label above this region in a radioautograph). The number of times each pattern was observed and the frequency this number represents is indicated at the right end of each horizontal line. The only restriction on the position of an individual pattern in the sequence is that it must be placed so that vertical columns of numbers are never broken. This restriction is imposed by the assumption that DNA synthesis in each chromosome region is uninterrupted. An example of a pattern which would not fit into the sequence shown in Table V is one in which only region 3 was labeled. A horizontal line bearing the number 3 could not be incorporated into the sequence at either end of the column of 3's without interrupting vertical columns of numbers.

The sequence into which the 13 X segment labeling patterns can be arranged makes an important prediction about the initiation of DNA synthesis in this chromosome. It can be seen in this array that labeling patterns which correspond to discontinuously labeled chromosomes occur at both the top and the bottom of the sequence. The labeling pattern corresponding to a continuously labeled chromosome must appear at an intermediate position in the sequence in order to satisfy the restriction that vertical columns of

TABLE V

Ordered Array of the Labeling Patterns Observed in the X-Chromosome Segment

X Segment	No. observed	Absolute frequency
——————7———	43	0.080
-1——————7———	9	0.017
*-1——————7——9-	56	0.105
-1——3——5——7——9-	4	0.007
-1——3——5——7-8-9-	5	0.009
-1——3——5-6-7-8-9-	20	0.037
-1-2-3——5-6-7-8-9-	4	0.007
-1-2-3-4-5-6-7-8-9-	51	0.095
-1————5——7-8-9-	1	0.002
-1————5——7——9-	5	0.009
*-1——————9-	6	0.011
-1———————	5	0.009
———————	326	0.609
	535	0.997

Each horizontal line represents an X-chromosome segment. Each number on the line represents labeling in the cytological region which bears that number. The array of patterns is constructed so that vertical columns of numbers are uninterrupted (see text). The patterns between the two asterisks have alternative positions in the array. Patterns shown above and below the asterisks, with the exception of (-), must appear at opposite ends of the sequence. The frequency with which each individual pattern was observed is indicated at the right end of each horizontal line.

numbers remain uninterrupted. If the vertical axis of the array of patterns is thought of as a time axis, then DNA synthesis would begin either at the top or at the bottom of the array. In either case, it is predicted that DNA synthesis does not start or stop simultaneously in all chromosome regions, but that there is a period during a cycle of chromosome duplication when synthesis occurs in all resolvable regions. (This is not meant to imply that all chromosomal DNA molecules which are capable of independent synthesis are necessarily replicating simultaneously in a continuously-labeled chromosome.) Beyond this statement, more precise details of DNA synthesis among the regions in the X segment cannot be predicted by the model array of labeling patterns. There are several reasons for this. First of all, it is impossible to tell from the available data which end of the sequence represents "start" and which "stop." Another source of uncertainty is that Table V represents only one of several slightly different arrays into which the labeling patterns can be arranged without interrupting vertical columns of numbers. Some of the labeling patterns which occur between the two asterisks along the left edge of the array have several alternative vertical positions in the sequence. Several of them could be positioned elsewhere or could appear more than once between the two asterisks. For example, pattern (1, 5, 7, 9) which appears just below pattern (1, 5, 7, 8, 9) could also appear directly beneath pattern (1, 7, 9), and no vertical columns of numbers would be interrupted. It could appear here exclusively or in both positions. Similarly, if the vertical order of all the patterns between the two asterisks were reversed, i.e. (1, 5, 7, 9) follows (1, 7), (1, 5, 7, 8, 9) follows (1, 5, 7, 9) etc., no vertical columns of numbers would be interrupted. However, all of these alternative sequences are similar in that they predict the initiation and termination of chromosomal DNA synthesis in only a few chromosome regions.

It must be stressed at this point that caution should be exercised in interpreting the array of patterns shown in Table V quantitatively. Although the frequency with which each labeling pattern appears in the array bears some relationship to the length of time spent by the chromosome in that portion of the S period represented by the pattern, the frequency is also a function of the ease with which the pattern can be unambiguously identified in radioautographs. Thus, patterns with a few labeled regions such as (1), (1, 7, 9), etc. can be easily identified. The same is true for chromosomes in which all regions are labeled (continuous pattern). However, in chromosomes which contain many labeled regions, yet are not continuously labeled, the frequency with which positive identification of the labeling pattern can be made is relatively low. Thus, the actual frequency of occurrence of patterns such as (1, 3, 5, 7, 8, 9) is probably higher than is shown in Table V. For this reason we do not suggest that the frequency of each labeling pattern can be directly translated into precise time intervals.

The single assumption of uninterrupted DNA synthesis in each chromosome region makes it possible to arrange all but one of the labeling patterns seen in chromosome III L in an array which is qualitatively similar to that generated

TABLE VI

Ordered Array of the Labeling Patterns Observed in the III L-Chromosome Segment

	No. observed	Absolute frequency
——————————9———	42	0.055
——————————9——11–	4	0.005
————————7——9——11–	9	0.012
————4———7–8–9——11–	1	0.001
——2——4–5——7–8–9——11–	2	0.003
*–1–2——4–5–6–7–8–9——11–	6	0.008
–1–2–3–4–5–6–7–8–9——11–	12	0.016
–1–2–3–4–5–6–7–8–9–10–11–	49	0.064
–1–2——4–5——7–8–9——11–	9	0.012
–1–2———5——7–8–9——11–	1	0.001
–1–2———5——7——9——11–	1	0.001
*–1————5——7——9——11–	2	0.003
–1————————7——9——11–	7	0.009
–1——————————9——11–	3	0.004
–1———————————	28	0.037
———————————	583	0.767
?————————7————	1	0.001
	760	0.999

See Table V for significance of asterisks.

TABLE VII

Ordered Array of Labeling Patterns Observed in the II R-Chromosome Segment

II R Segment		
	No. observed	Absolute frequency
–1———————	50	0.051
–1——3————	172	0.176
–1——3————8–	93	0.095
–1——3———6——8–	23	0.024
–1–2–3–4——6–7–8–	8	0.008
–1–2–3–4–5–6–7–8–	51	0.052
———————	578	0.593
	975	0.999

for the X segment (Table VI). The seven labeling patterns seen in the II R segment can also be ordered according to this assumption (Table VII). With regard to this chromosome, it is possible to place the continuous pattern at one end of the sequence without interrupting vertical columns of numbers. However, sequence analysis of combinations of labeling patterns seen in more than one chromosome in single nuclei suggests that DNA synthesis in the II R segment also begins and ends at only a few loci.

Table VIII shows an array of all the three-way combinations of labeling patterns which were observed in II R, III L, and the X segment. This multiple sequence is built in the same way as the individual sequences by assuming only that DNA synthesis in each chromosome region is uninterrupted during one cycle of chromosome duplication. Each three-way combination of patterns is inserted into the sequence as a unit so that vertical columns of numbers are not interrupted. One combination of patterns, indicated by a question mark, does not fit into the sequence. As in the individual sequences, patterns between the two asterisks have alternative positions in this portion of the array. It can be seen that the composite array has the same general property as the individual sequences for X and III L. Patterns corresponding to discontinuously labeled chromosomes must occur at both ends of the array in order to satisfy the restrictions on the order of patterns within the sequences for individual chromosomes. In particular, it is obvious that a II R sequence which has a continuous pattern at one end, could not fit into the composite sequence; e.g. triplet (1, 3)–(9)–(1, 7) and triplet (1, 3)–(1)–(1, 4, 7, 9) must occur at opposite ends of the composite sequence if vertical columns of numbers are to remain uninterrupted (the patterns are given in the order II R, III L, and X). Thus, the model composite array into which the observed labeling pattern combinations can be arranged predicts that DNA synthesis in all three chromosome segments studied begins and ends at only a few loci. It also predicts that although DNA synthesis does not begin or end simultaneously in all three chromosome segments, there is a period during an intermediate portion of S when synthesis occurs in all regions in all chromosomes.

The Assumption of Uninterrupted DNA Synthesis in Each Chromosome Region

Before the model sequence of polytene chromosome DNA synthesis is discussed further, the assumption on which it is built should be examined. In bacteria it has been shown that the single molecule genophore replicates as a unit or "replicon" (Jacob and Brenner, 1963). Clark and Maaløe (1967) have reported that the rate of DNA syn-

TABLE VIII

Ordered Array of the Triplets of Labeling Patterns Observed in the II R, III L, and X-Chromosome Segments in Single Nuclei

II R	III L	X	No.	Frequency
-1-	-	-	6	0.030
-1-	-	-7-	3	0.015
-1-3-	-	-7-	12	0.061
-1-3-	-9-	-1-7-	1	0.005
-1-3-	-9-	-1-7-9-	2	0.010
-1-3-8-	-9-	-1-7-9-	1	0.005
-1-3-8-	-7-9-11-	-1-7-9-	1	0.005
*-1-2-3-4-6-7-8-	-1-2-4-5-6-7-8-9-11-	-1-3-5-7-8-9-	1	0.005
-1-2-3-4-6-7-8-	-1-2-4-5-6-7-8-9-11-	-1-3-5-6-7-8-9-	1	0.005
-1-2-3-4-5-6-7-8-	-1-2-3-4-5-6-7-8-9-11-	-1-3-5-6-7-8-9-	1	0.005
-1-2-3-4-5-6-7-8-	-1-2-3-4-5-6-7-8-9-10-11-	-1-3-5-6-7-8-9-	1	0.005
-1-2-3-4-5-6-7-8-	-1-2-3-4-5-6-7-8-9-10-11-	-1-2-3-4-5-6-7-8-9-	47	0.238
-1-3-6-8-	-1-2-3-4-5-6-7-8-9-11-	-1-3-5-6-7-8-9-	3	0.015
-1-3-8-	-1-5-7-9-11-	-1-3-5-7-8-9-	1	0.005
-1-3-8-	-1-7-9-11-	-1-5-7-9-	1	0.005
*-1-3-8-	-1-	-1-7-9-	2	0.010
-1-3-	-1-	-1-7-9-	2	0.010
-1-3-	-	-1-7-9-	3	0.015
-1-3-	-	-1-9-	3	0.015
-1-3-	-	-1-	1	0.005
-1-3-	-	-	7	0.036
-	-	-	96	0.487
?-1-3-8-	-	-7-	1	0.005
			197	0.997

The sequence is built in the same way as those shown in Tables V–VII except that here triplets of patterns are inserted into the array as a unit.

thesis in the bacterial replicon is constant during a cycle of duplication. In the absence of evidence to the contrary, it seems reasonable to assume that DNA synthesis is continuous in chromosome loci which appear in radioautographs to be single units of DNA synthesis and may thus bear operational similarity to bacterial replicons (Plaut and Nash, 1964). The possibility must be considered, however, that chromosome regions which appear as units of DNA synthesis in radioautographs might in fact contain several units of synthesis (unresolvable by radioautography) among which replication is coordinated and continuous during a cycle of chromosome duplication. If this is the case, the assumption of uninterrupted DNA synthesis in chromosome regions is not invalidated. However, any sequence of chromosomal DNA synthesis built on this assumption must then become a model for DNA synthesis among groups of chromosome replication units (several in each region) rather than among individual units.

Comparison of the Model Sequence with Other Models of Polytene Chromosome DNA Synthesis

All models which have been proposed for the time course of DNA synthesis in dipteran polytene chromosomes, including the one presented in this paper, predict that DNA synthesis ends at scattered chromosome loci. (Keyl and Pelling, 1963; Gabrusewycz-Garcia, 1964; Fujita, 1965; Plaut et al., 1966; Berendes, 1966; Nash and Bell, 1968; Rodman, 1968). There is not general agreement, however, concerning the pattern of initiation of DNA synthesis in these chromosomes. Keyl and Pelling (1963) studied DNA synthesis in the polytene chromosomes of *Chironomus* using double labeling with ^{3}H and thymidine-^{14}C. On the some-

what dubious assumption that it is possible to distinguish accurately in a single layer of emulsion between silver grains produced by tritium and ^{14}C beta particles, the data obtained were interpreted as evidence that DNA synthesis in *Chironomus* polytene chromosomes begins simultaneously in all bands. In *Sciara*, on the other hand, Gabrusewycz-Garcia (1964) concluded from a radioautographic analysis of tritiated thymidine labeling patterns that DNA replication is initiated first in bands which will ultimately puff. This period was to be followed by one in which all bands are engaged in DNA synthesis. Finally, at the end of the DNA synthetic period, only heterochromatic bands were thought to be replicating.

Nash and Bell (1968) and Rodman (1968) have studied DNA synthesis in the polytene chromosomes of *Drosophila melanogaster* during late larval and prepupal stages. It is assumed in these studies that the last polytenic replication occurs during the period of experimentation and that no new cycles of chromosome duplication are initiated. It is shown that continuously labeled chromosomes are seen only during early portions of this period while discontinuous labeling patterns with small numbers of labeled chromosome regions are the only kind of patterns seen at the end of the period. One conclusion drawn from these observations is that a cycle of chromosome duplication ends with DNA synthesis at only a few chromosome loci. This is in complete accord with the model replication sequence being presented in this paper. The fact that chromosomal DNA replication may end with discontinuous labeling does not necessarily imply, however, that DNA synthesis begins with continuous labeling (Rodman, 1968). It should be noted that the earlier suggestion from our laboratory (Plaut et al., 1966), that continuous labeling patterns represent the initial phase of DNA synthesis, was also based on discontinuous labeling as the most probable terminal pattern and not on direct evidence. Our present model suggests that the continuous period of labeling does not occur at the very beginning of DNA synthesis in the polytene chromosome. It does not rule out the possibility suggested by Nash and Bell (1968) that simultaneous DNA synthesis at all loci might occur soon after the onset of chromosomal replication. If this were the case, one would still find continuous labeling patterns confined to the early portions of a cycle of chromosome duplication. At this point, a clear distinction should be made between a continuous labeling pattern as defined radioautographically, and simultaneous synthesis in all chromosomal DNA molecules which are capable of independent replication. Available techniques do not permit ruling out the possibility that in a continuously labeled chromosome there are non-replicating chromosome regions which, although present and unlabeled, are not revealed by the radioautographic method. Accordingly, there may never be a period during a cycle of chromosome duplication when all chromosomal DNA molecules are engaged in synthesis. The model replication sequence presented in this paper suggests that the period when polytene chromosomes exhibit a continuous labeling *pattern* occurs at an intermediate time during a cycle of chromosome duplication.

There is another aspect of DNA synthesis in the polytene chromosomes of *Drosophila melanogaster* which deserves comment. Berendes (1966) has proposed that certain bands in the male polytene X-chromosomes of *Drosophila melanogaster* replicate faster than their homologues in female X-chromosomes. However, the sequence of synthesis is thought to be the same. These conclusions are based on the observation that the number of labeled bands in male X-chromosomes was consistently less than the number of labeled bands in female X-chromosomes when pulse-labeled cells from each sex were observed at what was assumed to be the same time point in S. On the further assumption that DNA synthesis in polytene chromosomes begins simultaneously in all bands and ends with only a few bands replicating, it followed that the male X-chromosomes were farther advanced in DNA synthesis than were the female X-chromosomes at this particular moment in S. The criterion used to identify a definite time point was the appearance of a certain labeling pattern in an autosome. All nuclei in which autosome II R exhibited this pattern were presumed to be at the same time point in S. However, data obtained in our study and summarized in the model replication sequence (Table VIII) suggest that this assumption is invalid. It can be seen in Table VIII that patterns such as (1, 3) and (1, 3, 8) appear both above and below the continuous pattern in the II R portion of the composite array. If the vertical axis of this array is regarded as a time axis, then there would appear to be two non-contiguous portions of the DNA synthetic period in chromosome segment II R when pulses of

tritiated thymidine could produce the same labeling pattern. This means that one cannot assume that all nuclei which exhibit the same autosomal labeling pattern are in the same portion of the S period. (It must be emphasized that the observation of discontinuity in the appearance of labeling patterns does not imply discontinuity of DNA replication in individual chromosome regions; see Table VIII). Another observation from Table VIII is that during the two portions of the S period when regions 1 and 3 in the II R segment are replicating alone, producing pattern (1, 3) in radioautographs, the number of replicating regions in the X segment changes. Thus, pattern (1, 3) in II R can be found in association with X patterns (7), (1, 7), and (1, 7, 9) at one end of the model sequence and with X patterns (−), (1), (1, 7), and (1, 7, 9) at the other end of the sequence. This implies that the time periods during which chromosome segment II R exhibits labeling pattern (1, 3) are extended relative to the periods during which the X exhibits the patterns mentioned above. Therefore, it cannot be assumed that the appearance of an autosomal labeling pattern necessarily represents a particular *moment* in S. We conclude, on the basis of these observations and arguments that the existence of a fast replicating male X-chromosome cannot be demonstrated in *Drosophila melanogaster* with the criteria described by Berendes.

Implications of the Model Sequence for the Control of Chromosomal DNA Synthesis

Since so little is known about the factors involved in the control of chromosomal DNA synthesis, there is only limited value in speculating at length about the possible kinds of regulatory mechanisms which might be responsible for the time sequence of DNA synthesis envisioned in our model. However, it should be pointed out that the kind of control responsible for the initiation of DNA synthesis at a few loci may be much more complex than that which would have to be invoked for the simultaneous onset of DNA synthesis at all chromosome loci. In the latter case, a single stimulus to begin chromosomal DNA duplication could conceivably be all that is necessary to initiate DNA synthesis at all points along the chromosome. Such a stimulus could be either positive, e.g. the appearance of an initiator substance, or negative, e.g. the removal of an inhibitor, or both. In a situation where DNA synthesis begins at only a few loci, as is suggested in our model, the stimulus to start the S period may be effective in only a few chromosome regions. Additional stimuli might then be necessary to cause the onset of DNA synthesis at later times in other regions. On the other hand, in the *Drosophila* system there may be only a single stimulus to which different regions respond differentially in time. Another possibility is that there is a unique stimulus for each region, (Jacob and Brenner, 1963). In this case, ordered synthesis among replicons would reflect ordered production of stimuli. Available data do not allow a choice between these possibilities. Furthermore, it is unlikely that these are the only possible mechanisms of initiation which could be envisioned. At any rate, it is quite possible that once DNA replication begins in a region, its duration and eventual cessation may be a simple function of DNA content. Thus, the synthesis of DNA might stop when all of it has been duplicated once. Assuming a constant rate of synthesis, those regions having the greatest DNA content or the largest DNA molecules should spend the longest time in synthesis. There are no data presently available on the DNA content of the regions observed in our study. However, it can be pointed out that those regions which are largest and might be expected to contain the most DNA do not always exhibit the highest labeling frequencies. For example, in the II R segment, (Table I), region 1 is considerably smaller than region 4, yet the labeling frequency of region 1 is 6.7 times that of region 4 (Table I). Of course, it is possible that due to a greater degree of coiling or compaction of DNA, region 1 has a greater DNA content than does region 4. Whether or not this is the case will have to be determined spectrophotometrically.

Another possible aspect of control is that some chromosome regions might be heterochromatic (Heitz, 1928) and would then be expected to replicate late in S (Hsu et al., 1964; Gabrusewycz-Garcia, 1964), or begin replication with euchromatic regions but spend the longest time in synthesis (Keyl and Pelling, 1963), or exhibit some other sort of distinctive replicative behavior (Rudkin, 1963; Nur, 1966). However, in the model sequence in Table VIII, there is no region in any chromosome segment which is replicated exclusively at one end of the S period. Thus, there is no region that is either completely early or late replicating Also,. there is no indication that regions which spend the

longest time in synthesis, i.e. exhibit the highest labeling frequencies, are all replicated late in the sequence. Therefore, there is no reason to accept or reject at this time the possibility that heterochromatization plays a role in the control of chromosomal DNA synthesis in *Drosophila melanogaster*. However, the fact that replication of some heterochromatin, the chromocenter, is coordinated with that in the chromosome arms is indicated by our observation that no nucleus with labeled chromosome segments ever contains an unlabeled chromocenter. However, 32 nuclei were seen in which the chromocenter was labeled, but all regions in the three chromosome segments were unlabeled. It is concluded from this that the time span of DNA synthesis in the chromocenter is longer than that in the chromosome arms. There are three possible ways in which such an overlap could occur. First, DNA synthesis in the chromocenter could start before and end after DNA synthesis in the chromosome arms. Second, DNA synthesis could start first in the chromocenter but end simultaneously in the chromocenter and in some chromosome regions. Finally, replication in the chromocenter and in some chromosome regions could begin simultaneously but end last in the chromocenter. We have no data which indicate a choice between these three alternatives. In view of the already mentioned characterization of heterochromatin as late replicating, the third hypothesis might seem the most attractive.

This research was supported by a National Institutes of Health Research grant (CA-03276) and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

Received for publication 10 June 1968, and in revised form 11 July 1968.

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