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This thesis for the Master of Science Degree

by

Eileen Robertson

has been approved for the

Division of Natural Sciences

Incarnate Word College

by

Sister Catalina Iriarte, I.W.V.

Major Professor

Sister Joseph Marie Ramos

Committee Member

Sr. Jean Durel

Committee Member

San Antonio, Texas

February, 1981

COMPARISON OF DNA SYNTHESIS PATTERNS IN POLYTENE CHROMOSOMES BETWEEN NORMAL AND 20-HYDROXYECDYSONE-TREATED LARVAE

by

EILEEN ROBERTSON, B.A.

MASTERS THESIS

Presented to the Faculty of the Graduate School of

Incarnate Word College

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Author
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INTRODUCTION

The initiation of DNA synthesis in Dipteran polytene chromosomes has been studied in both normal and hormonally-induced states. The studies based on cytological observations utilizing an autoradiographic technique have formulated two conflicting theories on the initiation of replication in polytene chromosomes. The conflict stems from the two types of labelled chromosomes observed in all of the studies, the "continuously" labelled chromosome and the "discontinuously" labelled ones. The "continuously" labelled chromosome is one which has incorporated radioactive thymidine along its entire length. Studies supporting the theory that polytene chromosomes initiate DNA synthesis in all sites simultaneously under a central control mechanism consider the "continuously" labelled chromosome to represent the initiation of DNA synthesis (Plaut, 1963; Keyl and Pelling, 1963; Plaut, Nash and Fanning, 1966; Nash and Bell, 1968). The "discontinuously" labelled chromosome is one in which the incorporation of radioactive thymidine is observed in discrete, and possibly non-adjacent sites. Several studies consider this pattern to represent initiation of DNA synthesis and the beginning of the S-phase (Gabrusewycz-Garcia, 1964; Howard and Plaut, 1968; Fresquez, 1976).

The first autoradiographic study proposing that the "continuously" labelled chromosome represented initiation of DNA synthesis was that of Plaut in 1963. The discontinuously labelled chromosomes, therefore, were assumed to represent termination of synthesis. Plaut interpreted the presence of discontinuous label as evidence for the discontinuity of DNA in the chromosome. He believed that "several molecular ends [were present] in the DNA complement along the axis of each chromosome arm."

In the same year Keyl and Pelling studied initiation of DNA synthesis by injecting two different radioactive precursors into a hybrid of Chironomus thumni thumni and Chironomus thumni piger. These investigators felt they could distinguish between the grains resulting from the two precursors. The [^{14}C]thymidine was injected first. Various time intervals were allowed to elapse before the [^{3}H]thymidine was injected. They reported that the majority of continuously labelled chromosomes showed incorporation of [^{14}C]thymidine only, although some of these patterns seemed to have incorporated both [^{14}C]- and [^{3}H]thymidine. They stated that the discontinuously labelled chromosomes were always labelled with [^{14}C]thymidine along with an equal or lower number of [^{3}H]thymidine labelled bands. They concluded, therefore, that the continuous pattern represented initiation and the discontinuous pattern termination.

In 1964 Gabrusewycz-Garcia studied the incorporation of tritiated thymidine in the polytene chromosomes of late

third instar Sciara coprophila. She observed two basic patterns. In pattern P she observed label in the pre-puff and puff sites and in other euchromatic sites. In pattern C label was observed only in the centromeric region. Her analysis included grain density "labeling profiles" [sic] and a statistical comparison of labelling frequencies of the regions analyzed. In order to "test the regularity and the significance of variations in grain density along [the] chromosomes," Gabrusewycz-Garcia divided the chromosomes into four groups according to the intensity of label. These four categories represented the relative grain density of each site. The histograms of map region versus relative grain density, or "labeling profiles," revealed that the peaks in profile P are represented mostly by puff sites, whereas the peaks in profile C are generally restricted to heterochromatic sites. Furthermore, the P and C profiles seemed to be mirror images. The statistical analysis showed that the labelling frequencies of the different sites was non-random. From this, Gabrusewycz-Garcia concluded that replication within a chromosome is initiated asynchronously, beginning in the euchromatin (pre-DNA puff) sites and terminating in the heterochromatin areas. At some intermediate stage in the S-period, the whole chromosome is replicating and would be continuously labelled.

The work with polytene chromosomes during the latter part of the 1960's showed that labelling was always nonrandom in the different bands. Therefore, the chromosomal, or labelling, patterns began to be placed in an ordered arrangement to

indicate the temporal replication process for each region (Plaut, Nash and Fanning, 1966; Nash and Bell, 1968; Howard and Plaut, 1968). The matrix constructed from these patterns suggested one single, ordered replication sequence for all polytene chromosomes. The conformation of the matrix was determined by the first, or initiating, pattern. This reflected whether the investigators supported simultaneous initiation or initiation in discrete regions. The matrices constructed by Plaut, Nash and Fanning (1966) and Nash and Bell (1968) began with the continuously labelled chromosome and showed termination in progressively fewer sites. The matrices of Howard and Plaut (1968) began with a discontinuously labelled chromosome. Each chromosomal pattern that followed the first showed an increasing number of labelled sites. The continuously labelled pattern occupied an intermediate stage in the synthesis cycle. Termination was also viewed as occurring in progressively fewer sites.

In the matrices constructed by Plaut, Nash and Fanning (1966) and Nash and Bell (1968) several patterns did not fit. These were designated "exceptional" patterns. The "exceptional" patterns were not used in the construction of the matrix.

In 1972 Rudkin reviewed the conclusions of the investigators on initiation of DNA replication in polytene chromosomes. He designated the two types of matrices for the polytene chromosomes as the Simultaneous Model (Plaut, Nash and Fanning, 1966; Nash and Bell, 1968) and the Cascade Model

(Howard and Plaut, 1968). Rudkin criticized the Howard and Plaut model saying that it had not been experimentally distinguished from the Nash and Bell Simultaneous Model. Furthermore, the data from both studies could not be placed into only two error-free series which would show an uninterrupted labelled or unlabelled sequence for each region.

The initiation of the final S-period in the salivary glands of insects occurring prior to pupation is known to be under hormonal control (Clark, 1970). In the fly Rhynchosciara this occurs at late fourth instar. However, DNA synthesis can be induced hormonally in young fourth instar Rhynchosciara (Stocker and Pavan, 1973; Fresquez, 1976, 1979) at a stage when DNA synthesis is normally at a minimum (Simões, 1967, 1970).

Fresquez (1976) studied initiation of DNA synthesis and chromosomal patterns in thirty-day old Rhynchosciara hollaenderi by inducing synthesis with 20-hydroxyecdysone. The frequency of labelled sites of chromosomal segments was used to place the chromosomal patterns in a matrix. Early synthesis showed a few or several sites labelled, followed at later stages with increasing numbers of labelled sites until all sites showed label and, thereafter, decreased in the number of labelled areas. Many different sequences could be constructed with the data. However, the majority of chromosomal patterns could not be placed in an ordered sequence. Fresquez concluded that initiation of synthesis begins in a few or many discrete regions, but there is not a single

ordered sequence of DNA synthesis for all nuclei as had been suggested by previous investigators.

The purpose of this study is to compare the patterns of chromosomal DNA synthesis during normal larval development with those patterns observed by Fresquez (1976) for hormonally-induced DNA synthesis. This will determine whether DNA synthesis responds in the same way in normal development as it does in the hormonally-induced state.

MATERIALS AND METHODS

1. Larvae

Rhynchosciara larvae are gregarious and can synchronize their development when different groups are placed in the same container. Therefore, two groups of female Rhynchosciara hollaenderi larvae, late 4^{th} instar, were mixed. Both groups, came from the same mating chamber and had the same hatch date. After synchronization, the larvae were again separated into two groups, one serving as the morning sample group and the other as the late afternoon sample group. Salivary gland squashes were prepared twice daily once net fibers were observed.

Chromosomal DNA synthesis patterns were analyzed only for the last replication cycle in order to minimize the influence of overlapping cycles of synthesis. Hence, chromosomal patterns are given for the 65^{th} to the 70^{th} days of larval life. By day 70, the larval-pupal molt had taken place as evidenced by the degeneration of the salivary glands and other modifications of larval tissues.

2. Experimental Procedure

Five larvae were selected for each of the two daily sample points. The samples were taken at the same time each

day, six hours apart. Larvae were etherized (45-60 s) and injected in the distal region with 2 μl of tritiated thymidine (sp ac 17 Ci/mM). Only larvae that did not lose hemolymph after injection were used. After a five minute pulse, the pair of salivary glands were removed and sectioned in a chilled solution of 95% ethanol-acetic acid (3:1). Only the S_1 sections of the glands were used. These were placed on a siliconized cover slip holding a drop of chilled acetic acid (45%). The salivary gland was minced, then the cover slip was picked up with a lightly albuminized slide. After squashing, the slides were immersed in liquid nitrogen and the cover slips removed. Tissue was dehydrated in 95% ethanol, air-dried, and later processed for autoradiography.

3. Autoradiography

The slides were placed overnight in 70% ethanol then rinsed in distilled water prior to processing. Two slides, placed back-to-back, were dipped in a solution of Kodak NTB-2 and distilled water (1:1) then placed on drying racks. Slides were put into open slide boxes to air-dry for two hours in an enclosed black chamber equipped with a fan. At the end of the drying period the slides were placed in black slide boxes containing a vial of Dri-rite. These boxes were sealed with tape, enclosed in aluminum foil, and placed in a refrigerator (4°C). Incubation was fourteen days. Slides were developed in Kodak D-19 and stained with Unna's polychrome methylene blue.

4. Analysis

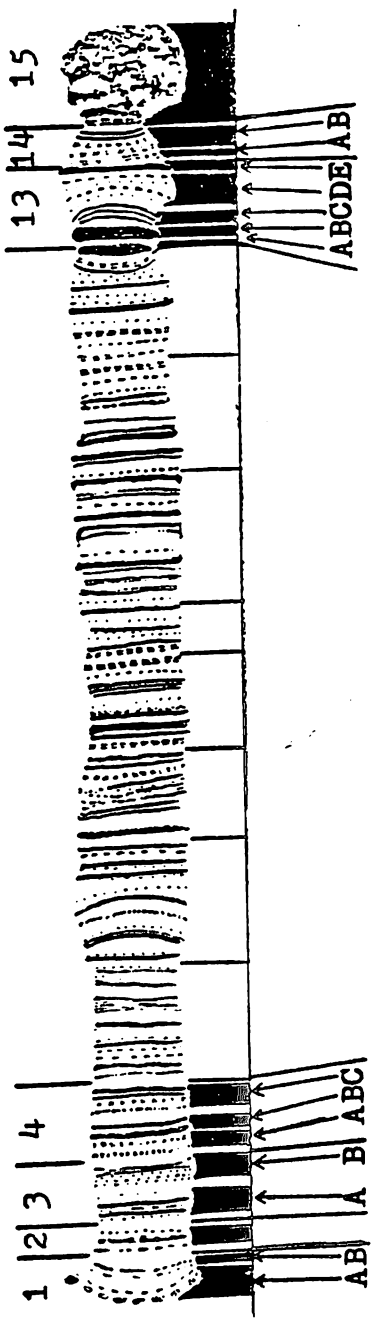
Two segments of the B chromosome were studied (see Figure 1). The regions analyzed were 1 to 4 and 13 to 15. These included two DNA puff-sites, 2 and 3A; four heterochromatic sites, 1A, 13A and B, and 15 (telomeric, intercalary, and centromeric heterochromatin, respectively); and several euchromatic regions.

A site was considered labelled when at least five grains of label, closely associated, were observed. A chromosome was counted as continuously labelled if six or less sites were unlabelled. In determining the percent of chromosomes labelled, Bartlett's correction factor of $(1/4n)(100\%)$ was introduced if no labelled chromosomes were observed, and $100 - \left[(1/4n)(100\%)\right]$ was used if all chromosomes observed were labelled (Steel and Torrie, 1960).

All discontinuously labelled chromosomes, except those in clusters, were recorded as chromosomal patterns. The chromosomal pattern is a set of plus (+) and minus (-) signs, where a plus sign represents a labelled site and a minus sign, a site in which no label was observed. These chromosomal patterns have been listed in Appendix II. The computer programs for the analysis of chromosomal data were written by Dr. John Ward.

FIGURE 1

Camera Lucida drawing of Chromosome B from the S_1 section of the salivary gland, from Rhynchosciara hollaenderi larvae. The regions analyzed are designated by the numbers; the sites by the letters.


15
14
13
ABCDE
AB
4
3
2
1
ABC
B
A
AB

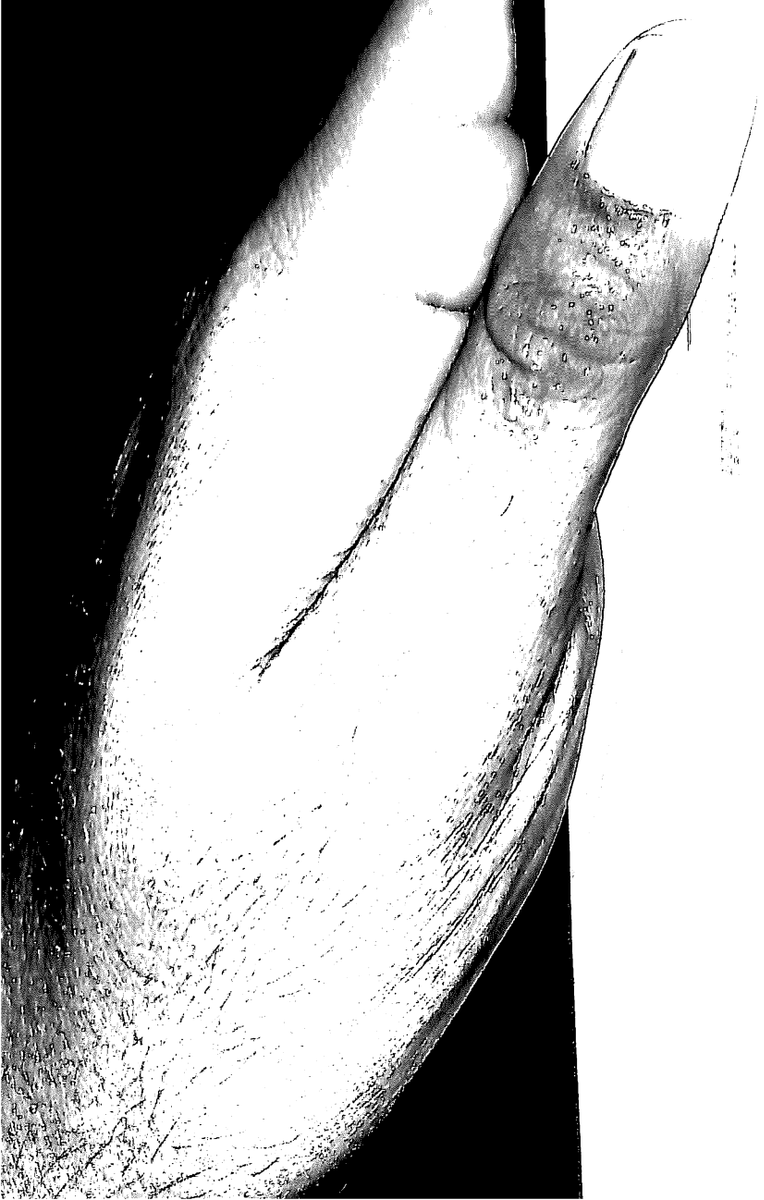
RESULTS

The total number of B chromosomes analyzed during the seven-day period prior to the formation of pupae was 4375. As explained in Material and Methods the samples were taken twice daily from two separate larval chambers. The data from the two daily samples were so much alike that they have been combined and are presented as a single sample point per day.

Figure 2 shows the incorporation of tritiated thymidine at the final DNA replication cycle of salivary gland chromosomes during the normal development of late fourth instar larvae. The figure shows the percent of labelled chromosomes for continuously labelled, discontinuously labelled, and their sum (total labelling observed).

A low amount of synthesis was observed the first two days as indicated by the approximately 10% labelled chromosomes. DNA synthesis began increasing on the third day with approximately 20% chromosomes labelled and followed on the fourth day with 31% of the chromosomes undergoing DNA replication. The highest percentage of DNA synthesis was reached the fifth day when 70% of the chromosomes were observed labelled. A sharp decrease in synthesis occurred on the sixth day and remained at that low level on the seventh day

FIGURE 2



Incorporation of [^{3}H]thymidine in chromosome B from salivary glands during final replication cycle of R. hollaenderi. Standard error (SE) not shown if less than or equal to one in order not to obscure symbols. Total label, ⬡—⬡; discontinuous label, □—□; continuous label, △—△.

FIGURE 2

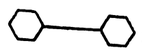
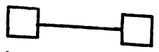
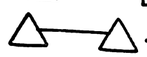
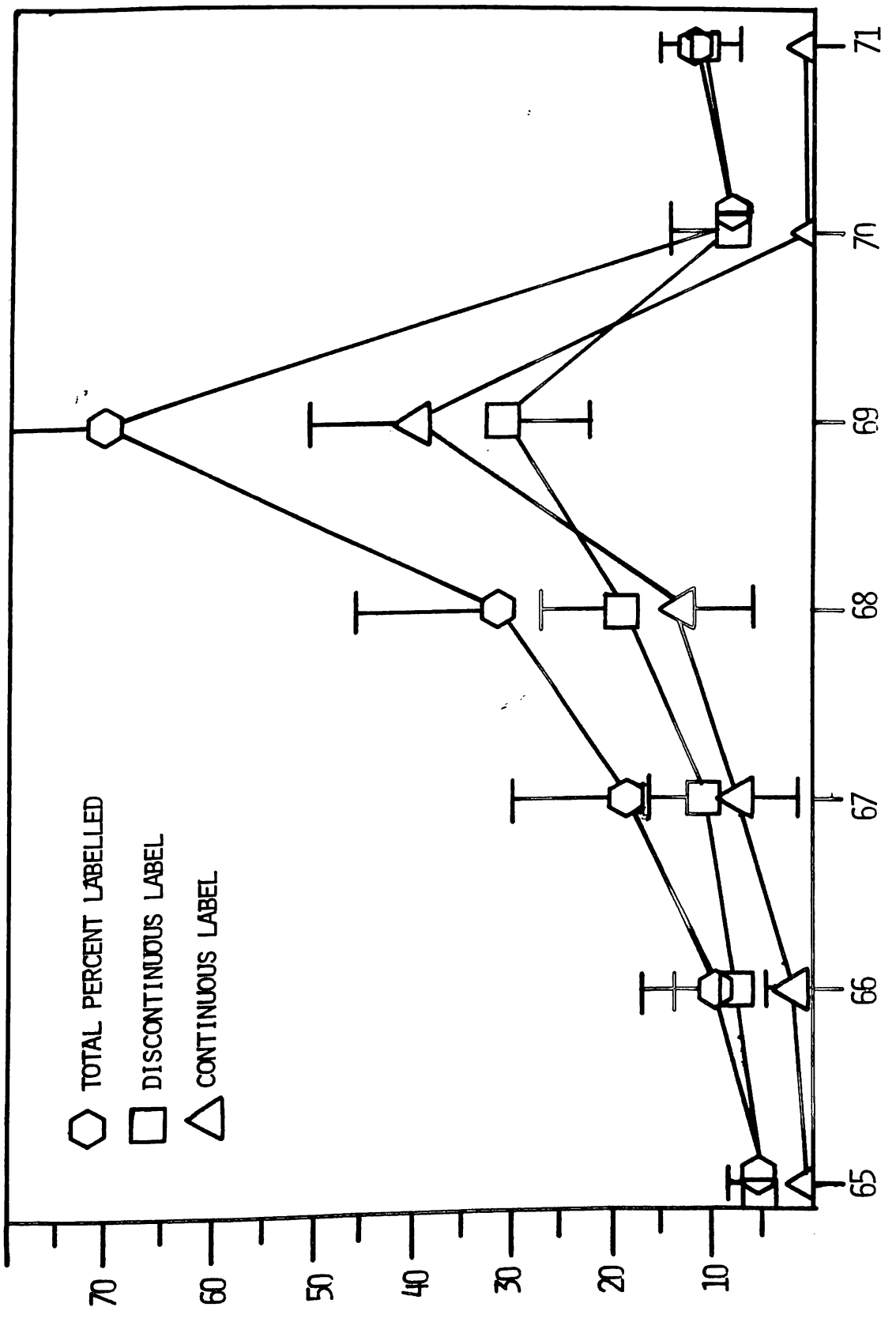
Incorporation of [^{3}H]thymidine in chromosome B from salivary glands during final replication cycle of R. hollaenderi. Standard error (SE) not shown if less than or equal to one in order not to obscure symbols. Total label,  ; discontinuous label,  ; continuous label,  .

FIGURE 2

Incorporation of [^{3}H]thymidine in chromosome B from salivary glands during final replication cycle of R. hollaenderi. Standard error (SE) not shown if less than or equal to one in order not to obscure symbols. Total label,  ; discontinuous label,  ; continuous label,  .


TOTAL PERCENT LABELLED
DISCONTINUOUS LABEL
CONTINUOUS LABEL
PERCENT LABELLED CHROMOSOMES
70
60
50
40
30
20
10
65
66
67
68
69
70
71
LARVAL AGE (DAYS)

(10%). On these days the chromosomes were observed to be in clusters, indicating that histolysis of the salivary glands had begun.

The increase in labelling for each day is reflected in both the discontinuously and continuously labelled chromosomes. The discontinuously labelled chromosomes constituted a majority from the first to the fourth days as well as the last two days of the experiment. The continuously labelled chromosomes were a majority only on the fifth day.

The first four days (65, 66, 67, and 68) show the percentage of discontinuously labelled chromosomes to have been 5%, 8%, 11%, and 18%, respectively, whereas continuously labelled were less than 1% on the first day, and 2%, 8%, and 13% on the following three days, respectively. No significant difference was indicated ($t_{(.05,18)} = 2.101$). The increase in discontinuously labelled chromosomes on the third and fourth days may simply be a reflection of the increase in the number of chromosomes incorporating label. On the fifth day (69), the percentage of continuously labelled was almost 40% while 31% were discontinuously labelled. There was no significant difference between the two classes of chromosomes ($t_{(.05,\ 20)} = 2.086$).

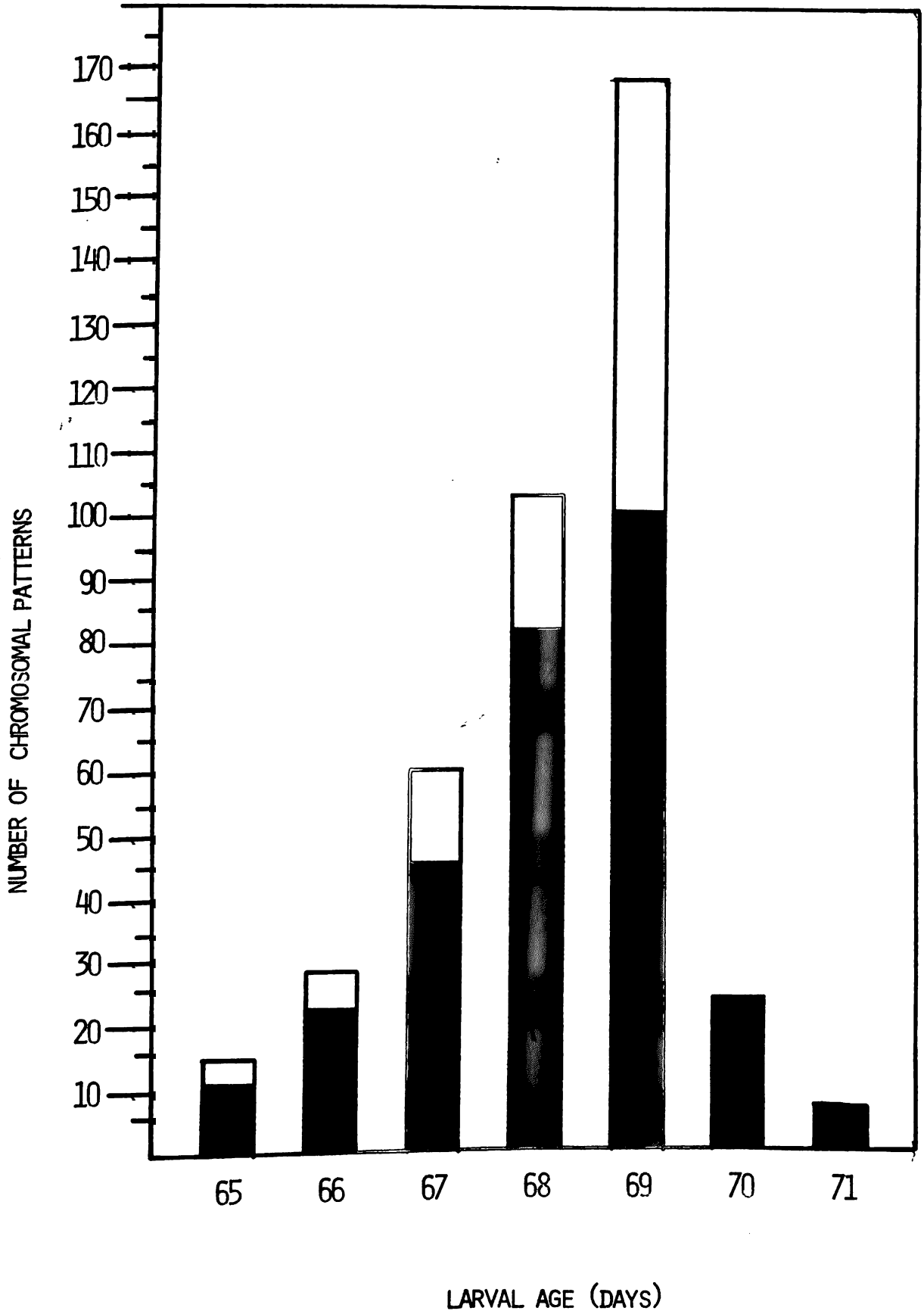
The total number of chromosomal patterns (discontinuously labelled chromosomes) is shown in Figure 3. The bars represent the actual number of patterns observed per day with the shaded portions indicating the unique patterns. The unique chromosomal patterns are patterns observed in only one

FIGURE 3

Total number of chromosomal patterns for final replication cycle of R. hollaenderi.

 designates difference between total number of patterns and unique patterns, frequency of repeated patterns;

 designates number of unique patterns.


NUMBER OF CHROMOSOMAL PATTERNS
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
65
66
67
68
69
70
71
LARVAL AGE (DAYS)

discontinuously labelled chromosome. Chromosomal patterns that have been recorded from two or more discontinuously labelled chromosomes are repeated patterns. The frequency of chromosomes per repeated pattern ranged from 2 to 22. Only 3 repeated patterns are represented in the higher range and these are labelled in a single site (e.g., site 2). On all days the number of patterns that were unique greatly exceeded the repeated.

The total number of discontinuously labelled chromosomes observed was 403. When the data was tallied for each of the days there were 290 unique and 33 repeated patterns. However, since a pattern which was unique on a given day sometimes occurred (unique or repeated) on another day, the data for all 7 days shows that only 236 patterns were unique while 44 (167 chromosomes) were repeated. In short, 280 patterns were recorded (Appendix II).

The chromosomal patterns from this experiment were compared with those of Fresquez' (1976) experiment B. Only 40 of her 177 patterns match with ones observed in this experiment. Twenty-four of these were among the patterns classed as unique and 16 among the repeated.

The relative labelling frequency for each of the 16 sites analyzed are listed in Table 1. The sites with the highest frequencies are the two DNA puff sites (2 and 3A) and the heterochromatin region associated with the telomeric centromere (region 15). The sites were arranged in a matrix in a descending order of frequency in keeping with previous

TABLE 1

Relative labelling frequency of 16 sites of chromosome B for the seven day final replication cycle.

§, heterochromatin; #, DNA puff site; other, euchromatin.

Total number of discontinuously labelled chromosomes: 403								
Location of Bands	1A[§]	1B	2[#]	3A[#]	3B	4A	4B	4C
Chromosomes Labelled in specific site	177	206	354	292	213	162	113	249
Relative Labelling frequency	0.439	0.511	0.878	0.725	0.528	0.402	0.280	0.618

Location of Bands	13A[§]	13B[§]	13C	13D	13E	14A	14B	15[§]
Chromosomes Labelled in specific site	62	89	162	88	102	133	180	306
Relative Labelling frequency	0.154	0.221	0.402	0.218	0.253	0.330	0.447	0.759

studies. Twenty-six matrices of varying lengths (5-24 patterns) were constructed, but no matrix could include more than 24 patterns. Thirteen of the matrices could incorporate 8 or more patterns. Once a pattern was placed in a matrix it was not used again. Only 25 patterns were not incorporated into any meaningful matrices. The largest matrix is shown in Table 2.

The relative labelling frequency can also be used to determine if each chromosomal pattern occurs with the frequency predicted by chance. The hypothesis is that if replication in each site is an independent event, then the probability of observing a specific pattern is equal to the product of the labelled/unlabelled frequency (probability) of each site, where the value of the probability is determined by whether the site is labelled or unlabelled. To obtain the probability of a site being unlabelled the relative labelling frequency was subtracted from one. With these probabilities and the chromosomal patterns, the data can be analyzed using the Chi-square test. In order to simplify the mathematics only three sites were considered, sites 1A, 1B, and 2. These sites included heterochromatin (1A), euchromatin (1B), and a DNA puff site (2). With 3 sites, 8 labelling patterns are possible. These patterns are shown in Table 3. The calculated Chi-square of 170.310 indicates that the hypothesis cannot be correct as will be explained in the discussion.

TABLE 2

Chromosomal matrix suggesting DNA replication process for a polytene chromosome.

Symbols same as for Table 1.

CHROMOSOMAL MATRIX

B CHROMOSOME SITES

PATTERN NUMBERS	1		2#	3		4			13					14		15§
	A§	B		A#	B	A	B	C	A§	B§	C	D	E	A	B	
264	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
244	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
186	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
105	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
103	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	+
87	+	+	+	+	+	-	-	+	-	-	-	-	-	+	-	+
83	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	+
52	+	+	+	+	+	+	-	+	-	-	+	-	+	+	+	+
50	+	+	+	+	+	+	-	+	-	-	+	-	+	+	+	+
24	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
	(continuously labelled chromosome)															
3	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
14	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
28	+	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+
30	+	+	+	+	+	+	+	+	-	-	+	-	-	+	+	+
54	+	+	+	+	+	+	-	+	-	-	+	-	-	+	+	+
60	+	+	+	+	+	+	-	+	-	-	-	-	-	+	+	+
86	+	+	+	+	+	-	-	+	-	-	-	-	-	+	+	+
169	-	+	+	+	+	-	-	+	-	-	-	-	-	+	+	+
215	-	-	+	+	+	-	-	+	-	-	-	-	-	+	+	+
233	-	-	+	+	-	-	-	+	-	-	-	-	-	-	+	+
234	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	+
243	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+
280	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

TABLE 3

Chi-square table. The frequency of observed and expected chromosomal patterns for 3 sites (1A, 1B, and 2) of the polytene chromosome.

SITES 1A	SITES 1B	SITES 2	Observed Frequency	Expected Frequency	(Obs. - Exp.) / Expected
+	-	-	2/403 = 0.005	0.026	6.860
+	+	-	0/403 = 0.000	0.027	10.881
+	+	+	141/403 = 0.350	0.197	47.810
-	+	+	58/403 = 0.143	0.252	18.681
-	-	+	121/403 = 0.300	0.241	5.870
-	-	-	40/403 = 0.099	0.033	53.609
+	-	+	34/403 = 0.084	0.188	23.021
-	+	-	7/403 = 0.017	0.035	3.578
				X^2 =	170.310

DISCUSSION

In normal development, DNA replication begins in the distal region (S_3) of the salivary glands of Rhynchosciara hollaenderi and steadily progresses to the proximal end (S_1) until 100% of the cells are synthesizing DNA (Simões, 1970). In this study the progression of cells entering the S-period in the S_1 section of the gland is reflected in the increasing frequency of labelled chromosomes from the 65th day through the 69th day. Although 100% labelling was not observed in this study, it was probably reached immediately before or after the 69th day, since the marked decrease observed at day 70 indicated termination had taken place. In addition, DNA puffs at site 2 of chromosome B were observed on days 68 and 69 just before histolysis of the salivary glands (day 70). Histolysis also indicates the end of the last replication cycle. The length of the replication cycle in this experiment agrees with that of Cordeiro and Meneghini (1973) who found that the final synthesizing cycle in R. angelae takes 5 to 6 days.

Each daily increase in total labelled chromosomes reflects the number of cells starting DNA synthesis. This also suggests a way to determine whether the initiation of DNA replication is continuous or discontinuous along the chromosome.

If replication begins in all sites of the chromosome,

then the highest percentage of continuously labelled chromosomes would have occurred at the beginning of the observation period. However, the results show that the continuously labelled chromosomes were a minority on the first four days. Only on the 69th day did the continuously labelled chromosomes form a majority. Since the discontinuously labelled chromosomes were the majority of labelled chromosomes the first 4 days, they probably all represent initiation of replication. By the fourth and fifth days (days 68 and 69) most chromosomes were in an intermediate S-phase as indicated by the increased frequency of continuously labelled chromosomes. The discontinuously labelled chromosomes observed on days 70 and 71 must represent termination since there was such a drastic decrease in the total number of labelled chromosomes after the 69th day.

The abundance of unique chromosomal patterns also suggests that initiation begins in separate sites of the chromosome. Large numbers of unique patterns would not be possible if replication began in all bands simultaneously. With simultaneous initiation, the discontinuous patterns would have to represent termination. Since chain elongation is known to proceed at the same rate (Callan, 1974; Laird, Chooi, Cohen, Dickson, Hutchinson, and Turner, 1974), the discontinuous patterns would have resulted from the increased length of time required for bands with a greater amount of DNA to replicate. In that case the repeated patterns would be expected in greater numbers. However, these are observed

less frequently than the unique patterns. Hence, the dearth of repeated patterns suggests that initiation most probably begins in the discontinuously labelled chromosomes.

The observation of matching patterns between this work and that of Fresquez (1976) indicates that hormone induction of DNA synthesis is similar to that observed during normal development. Investigations with induced synthesis can therefore be used to provide insights into the control of DNA synthesis.

As shown in the results, relatively few patterns were observed (280) in comparison with what would be expected if synthesis were random. Since 16 sites were studied, this should have resulted in 2^{16} = 65 536 possibilities. The low number of chromosomal patterns observed indicates DNA synthesis is non-random. Even these few patterns could not be arranged in a single ordered sequence. This agrees with the observations Fresquez (1976) presented on hormonally induced DNA synthesis. Consequently, the suggestion of other investigators (Plaut, Nash and Fanning, 1966; Nash and Bell, 1968; Howard and Plaut, 1968) that replication in polytene chromosomes follows a single ordered array cannot apply to Rhynchosciara.

The coordination of synthesis among different sites on the chromosomes was shown by the Chi-square value reported in Table 3. At the one percent level of significance and seven degrees of freedom, the critical Chi-square would be 18.475. The calculated Chi-square value, 170.310, is about 9 times greater than 18.475. Hence, the probability that the

labelling patterns resulted from a random association of labelled sites is exceedingly small. The hypothesis that replication in each site is an independent event has to be rejected.

Table 3 was also used to see if co-ordinated replication among the sites was based on their location along the chromosome. The physical continuity of the DNA molecule could cause co-ordinated replication at adjacent sites. If adjacent sites influence each other's replication then the first 5 patterns in Table 3 would be expected to be the process for replication along the chromosome. Pattern 6 is neutral, and the last two patterns, + - +, and - + -, are opposite and, therefore, not allowed. Of the allowed patterns, pattern 2, + + -, was not observed. Yet the two patterns that are disallowed were both observed several times. Both allowed and disallowed patterns cannot both exist if a single ordered sequence of replication is the rule. Physical continuity of the DNA molecule, therefore does not influence replication at adjacent sites, and some other control mechanism must be invoked.

The mechanism controlling initiation of replication at non-adjacent sites along the chromosome could depend on the physiological state of the cell at the time the S-period begins. Puffing patterns have been correlated with different physiological states (Mechelke, 1953; Berendes, 1965, 1973; Ashburner, 1970). The formation of a puff on the polytene chromosome is caused by decondensation of DNA and is a morphological manifestation of gene activity (Breuer and Pavan,

1952; Beermann, 1952). In partially synchronized cells, slight variations in gene activity would result if either different bands were puffed or if the same bands were puffed, but the degree of puffing varied. When these cells enter the S-period, the sites at which the DNA is uncoiled could be the target for initiation of DNA replication. This could account for the co-ordination of replication between sites that are not adjacent and the non-randomness of the various chromosomal patterns. The number of different matrices, therefore, simply reflects the different chromosomal replication processes. The possibility that DNA initiation occurs in the decondensed areas of the chromosome can readily be checked by determining whether the radioactive label lies exclusively over these areas (puffs and interbands) during the early part of the S-phase.

CONCLUSION

The initiation of DNA replication at the final S-cycle of normally developing Rhynchosciara hollaenderi agrees with the conclusions of Fresquez for hormonally-induced synthesis in the same organism. This confirms the validity of interpretations based on hormonally induced DNA synthesis and provides a predictable system for additional studies.

This work shows that:

1. Initiation of replication begins at separate sites of the polytene chromosome; and

2. the chromosomal replication process is co-ordinated among the sites, but in polytene chromosomes of the salivary glands there is more than one co-ordinated sequence for this process.

Since the sequences with which the sites entered the replication process could not be explained by their frequency or nearest-neighbor location, the co-ordination among the sites indicated some other mechanism controlled replication in different cells. The suggestion offered is that the physiological state of the cell at the time S-phase begins is the determining mechanism.

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APPENDICES

APPENDIX I

Raw Data for labelled and unlabelled chromosomes from days (larval ages) 65 to 71. Bartlett's correction factor, (1/4n)(100%) and 100 - [(1/4n)(100%)], was used when no labelling was observed or when all chromosomes were labelled, respectively (Steel and Torrie, 1960).

TABLE 1

PERCENT OF LABELLED CHROMOSOMES FROM S_1 SECTION OF SALIVARY GLANDS OF 65 DAY OLD LARVAE.

Gland	Unlabelled	LABELLED			Total Number Chromosomes Counted	% LABELLED		
		Continuous	Discontinuous	Total		Continuous	Discontinuous	Total
1	82	0	0	0	82	0.30	0.30	0.30
2	86	0	0	0	86	0.29	0.29	0.29
3	62	0	0	0	62	0.40	0.40	0.40
4	28	0	1	1	29	0.86	3.44	3.44
5	74	0	0	0	74	0.34	0.34	0.34
6	32	0	1	1	33	0.76	3.03	3.03
7	21	0	13	13	34	0.73	38.23	38.23
8	48	0	0	0	48	0.52	0.52	0.52
9	53	0	0	0	53	0.47	0.47	0.47
10	64	0	0	0	64	0.39	0.39	0.39
	550	0	15	15	565	5.06	47.41	47.41
					$\bar{x}$ =	0.51	4.74	4.74
					S.E. =	±0.06	±3.74	±3.74

TABLE 2

PERCENT OF LABELLED CHROMOSOMES FROM S_1 SECTION

OF SALIVARY GLANDS OF 66 DAY OLD LARVAE.

Gland	Unlabelled	LABELLED			Total Number Chromosomes Counted	% LABELLED		
		Continuous	Discontinuous	Total		Continuous	Discontinuous	Total
1	17	6	20	26	43	13.95	46.51	60.46
2	46	0	0	0	46	0.54	0.54	0.54
3	46	0	0	0	46	0.54	0.54	0.54
4	62	0	5	5	67	0.37	7.46	7.46
5	74	0	1	1	75	0.33	1.33	1.33
6	38	0	2	2	40	0.62	5.00	5.00
7	55	0	0	0	55	0.45	0.45	0.45
8	46	0	0	0	46	0.54	0.54	0.54
	384	6	28	34	418	17.34	62.37	76.32
					$\bar{x}$ =	2.17	7.80	9.54
					S.E. =	±1.68	±5.61	±7.33

TABLE 3

PERCENT OF LABELLED CHROMOSOMES FROM S_1 SECTION OF SALIVARY GLANDS OF 67 DAY OLD LARVAE.

Gland	Unlabelled	LABELLED			Total Number Chromosomes Counted	% LABELLED		
		Continuous	Discontinuous	Total		Continuous	Discontinuous	Total
1	83	0	0	0	83	0.30	0.30	0.30
2	80	0	0	0	80	0.31	0.31	0.31
3	62	0	0	0	62	0.40	0.40	0.40
4	58	0	4	4	62	0.40	6.45	6.45
5	73	0	2	2	75	0.33	2.67	2.67
6	0	42	27	69	69	60.87	39.13	99.64
7	76	0	2	2	78	0.32	2.56	2.56
8	75	0	0	0	75	0.33	0.33	0.33
9	15	6	25	31	46	13.04	54.35	67.39
10	39	0	0	0	39	0.64	0.64	0.64
	561	48	60	108	669	76.94	107.14	180.69
					$\bar{x}$ =	7.69	10.71	18.07
					SE =	±6.04	±6.14	±11.19

TABLE 4

PERCENT OF LABELLED CHROMOSOMES FROM S_1 SECTION OF SALIVARY GLANDS OF 68 DAY OLD LARVAE.

Gland	Unlabelled	LABELLED			Total Number Chromosomes Counted	% LABELLED		
		Continuous	Discontinuous	Total		Continuous	Discontinuous	Total
1	88	0	0	0	88	0.28	0.28	0.28
2	60	0	1	1	61	0.41	1.64	1.64
3	3	52	32	84	87	59.77	36.78	96.55
4	0	35	21	56	56	62.50	37.50	99.55
5	78	0	0	0	78	0.32	0.32	0.32
6	87	0	0	0	87	0.28	0.28	0.28
7	1	4	41	45	46	8.69	89.13	97.82
8	23	0	0	0	23	1.09	1.09	1.09
9	39	1	8	9	48	2.08	16.67	18.75
10	90	0	0	0	90	0.28	0.28	0.28
	469	92	103	195	664	135.70	183.97	316.56
					$\bar{x}$ =	13.57	18.40	31.65
					SE =	±7.97	±9.19	±14.58

TABLE 5

PERCENT OF LABELLED CHROMOSOMES FROM S_1 SECTION OF SALIVARY GLANDS OF 69 DAY OLD LARVAE.

Gland	Unlabelled	LABELLED			Total Number Chromosomes Counted	% LABELLED		
		Continuous	Discontinuous	Total		Continuous	Discontinuous	Total
1	35	0	9	9	44	0.56	20.45	20.45
2	11	6	27	33	44	13.63	61.36	75.00
3	0	88	3	91	91	96.70	3.30	99.72
4	5	1	16	17	22	4.54	72.73	77.27
5	103	0	4	4	107	0.23	3.73	3.73
6	0	62	8	70	70	88.57	11.43	99.64
7	0	20	32	52	52	38.46	61.54	99.52
8	6	19	44	63	69	27.53	63.76	91.30
9	0	49	14	63	63	77.78	22.22	99.60
10	0	78	10	88	88	88.63	11.36	99.72
11	34	0	4	4	38	0.66	10.52	10.52
	194	323	171	494	688	437.29	342.40	776.47
					$\bar{x}$ =	39.75	31.12	70.59
					SE =	±12.12	±8.28	±11.80

TABLE 6

PERCENT OF LABELLED CHROMOSOMES FROM S_1 SECTION OF SALIVARY GLANDS OF 70 DAY OLD LARVAE.

Gland	Unlabelled	LABELLED			Total Number Chromosomes Counted	% LABELLED		
		Continuous	Discontinuous	Total		Continuous	Discontinuous	Total
1	18	0	16	16	34	0.73	47.06	47.06
2	56	0	0	0	56	0.44	0.44	0.44
3	79	0	0	0	79	0.31	0.31	0.31
4	71	0	0	0	71	0.35	0.35	0.35
5	70	0	0	0	70	0.35	0.35	0.35
6	67	0	0	0	67	0.37	0.37	0.37
7	35	0	16	16	51	0.49	31.37	31.37
8	53	0	0	0	53	0.47	0.47	0.47
9	69	0	0	0	69	0.36	0.36	0.36
10	85	0	0	0	85	0.29	0.29	0.29
	603	0	32	32	635	4.16	81.37	81.37
					$\bar{x}$ =	0.41	8.13	8.13
					SE =	±0.04	±5.31	±5.31

TABLE 7

PERCENT OF LABELLED CHROMOSOMES FROM S_1 SECTION OF SALIVARY GLANDS OF 71 DAY OLD LARVAE.

Gland	Unlabelled	LABELLED			Total Number Chromosomes Counted	% LABELLED		
		Continuous	Discontinuous	Total		Continuous	Discontinuous	Total
1	50	0	14	14	64	0.39	21.87	21.87
2	77	0	0	0	77	0.32	0.32	0.32
3	75	0	2	2	77	0.32	2.59	2.59
4	92	0	11	11	103	0.24	10.68	10.68
5	64	0	8	8	72	0.34	11.11	11.11
6	69	0	0	0	69	0.36	0.36	0.36
7	91	1	0	1	92	1.09	0.27	1.09
8	82	0	2	2	84	0.30	2.38	2.38
9	28	0	10	10	38	0.65	26.32	26.32
10	39	0	21	21	60	0.42	35.00	35.00
	667	1	68	69	736	4.43	110.90	111.72
					$\bar{x}$ =	0.44	11.09	11.17
					SE =	±0.08	±3.97	±3.94

APPENDIX II

Chromosomal DNA synthesis patterns for 16 sites of Chromosome B. Pattern numbers assigned arbitrarily. The patterns matching those of Fresquez (1976) are indicated by asterisk (*). The number in parenthesis is the number of patterns repeated for the total 7 day replication cycle. Sites analyzed include heterochromatin ($), DNA puff sites (#), and euchromatin. The plus (+) sign indicates a site labelled with [^{3}H]thymidine; a minus (-) sign indicates an unlabelled site.

CHROMOSOMAL PATTERNS

Pattern Numbers (arbitrary) \ Sites analyzed	1		2#	3		4			13					14		15§
	A§	B		A#	B	A	B	C	A§	B§	C	D	E	A	B	
1	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
*2	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+
*3	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+
*5	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
6	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-
*7 (2)	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+
*9	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+
10	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	-
11	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+
*12 (2)	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+
*14 (4)	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
*15	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+
16 (2)	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+
*17	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+
*18 (3)	+	+	+	+	+	+	+	+	-	+	+	-	-	+	-	+
*19	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+
20	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+
21	+	+	+	+	+	+	+	+	-	+	-	+	-	-	+	+
22	+	+	+	+	+	+	+	+	-	+	-	-	+	+	+	+
23	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+
*24 (5)	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+
*26 (2)	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+
*27	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+
*28 (7)	+	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+
*29	+	+	+	+	+	+	+	+	-	-	+	-	+	-	+	+
30 (2)	+	+	+	+	+	+	+	+	-	-	+	-	-	+	+	+
*31	+	+	+	+	+	+	+	+	-	-	+	-	-	-	+	+
*32	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+
33	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	+
34	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+

(Continued on next page)

Pattern Numbers (arbitrary) \ Sites analyzed	1 A§B	2#	3 A#B	4 A B C	13 A§B§C D E	14 A B	15§
35	+ +	+	+ +	+ + -	+ + - - -	+ +	+
36	+ +	+	+ +	+ - +	+ + + + +	+ +	+
37	+ +	+	+ +	+ - +	+ + + + -	- -	+
38	+ +	+	+ +	+ - +	+ + - + +	+ +	+
39 (2)	+ +	+	+ +	+ - +	+ + - - +	+ +	+
40	+ +	+	+ +	+ - +	+ - + + +	+ -	+
41	+ +	+	+ +	+ - +	+ - + - +	+ +	+
*42	+ +	+	+ +	+ - +	+ - + - -	+ +	+
43	+ +	+	+ +	+ - +	+ - - + +	+ -	+
*44	+ +	+	+ +	+ - +	- + + + +	+ +	+
45	+ +	+	+ +	+ - +	- + + + +	- +	+
46	+ +	+	+ +	+ - +	- + + + +	- -	+
47	+ +	+	+ +	+ - +	- + + - -	+ +	+
48 (2)	+ +	+	+ +	+ - +	- + - - +	- +	+
49	+ +	+	+ +	+ - +	- + - - -	+ +	+
*50 (4)	+ +	+	+ +	+ - +	- - + - +	+ +	+
51	+ +	+	+ +	+ - +	- - + + -	+ +	+
52 (2)	+ +	+	+ +	+ - +	- - + - +	+ +	+
53	+ +	+	+ +	+ - +	- - + - +	- +	+
*54	+ +	+	+ +	+ - +	- - + - -	+ +	+
*55 (2)	+ +	+	+ +	+ - +	- - + - -	- -	+
56	+ +	+	+ +	+ - +	- - + - -	- -	-
57	+ +	+	+ +	+ - +	- - - + +	+ +	+
58	+ +	+	+ +	+ - +	- - - + -	+ +	+
59	+ +	+	+ +	+ - +	- - - - +	+ -	+
60	+ +	+	+ +	+ - +	- - - - -	+ +	+
61	+ +	+	+ +	+ - -	+ + + + -	+ +	+
62	+ +	+	+ +	+ - -	- + - - -	+ -	+
*63	+ +	+	+ +	- + +	+ + + + +	+ +	+
64	+ +	+	+ +	- + +	+ - + - +	+ +	+
65	+ +	+	+ +	- + +	- + + + +	+ +	+
*66	+ +	+	+ +	- + +	- - + + +	+ +	+
*67	+ +	+	+ +	- + +	- - + - +	+ +	+
68	+ +	+	+ +	- + +	- - + - +	+ -	+
69	+ +	+	+ +	- + +	- - + - +	- +	+
70	+ +	+	+ +	- + +	- - - - -	+ +	+

(Continued on next page)

Pattern Numbers (arbitrary) \ Sites analyzed	1 A§B	2#	3 A#B	4 A B C	13 A§B§C D E	14 A B	15§
71	+ +	+	+ +	- - +	+ + - - +	- +	+
72	+ +	+	+ +	- - +	+ - + - +	+ -	-
73	+ +	+	+ +	- - +	+ - - + -	- +	+
74	+ +	+	+ +	- - +	+ - - - -	+ +	+
75	+ +	+	+ +	- - +	- + + + -	+ +	+
76	+ +	+	+ +	- - +	- + + - +	+ +	+
77	+ +	+	+ +	- - +	- + - + -	+ +	+
78 (2)	+ +	+	+ +	- - +	- + - - -	- +	+
79	+ +	+	+ +	- - +	- - + + -	- +	+
80	+ +	+	+ +	- - +	- - + - +	+ +	-
81	+ +	+	+ +	- - +	- - + - +	- +	+
82 (3)	+ +	+	+ +	- - +	- - + - -	+ +	+
83 (2)	+ +	+	+ +	- - +	- - + - -	+ -	+
84	+ +	+	+ +	- - +	- - + - -	- +	+
85	+ +	+	+ +	- - +	- - + - -	- -	-
86	+ +	+	+ +	- - +	- - - - -	+ +	+
87	+ +	+	+ +	- - +	- - - - -	+ -	+
88	+ +	+	+ +	- - -	+ - + - -	+ +	+
89	+ +	+	+ +	- - -	+ - - - -	+ +	+
90	+ +	+	+ +	- - -	- - + + +	- +	+
91	+ +	+	+ -	+ + +	+ - - + +	+ +	+
92	+ +	+	+ -	+ + +	- + + + -	+ -	+
93	+ +	+	+ -	+ - +	+ - + - -	+ +	-
94	+ +	+	+ -	+ - +	- - + + -	- +	+
95	+ +	+	+ -	+ - +	- - + + -	- -	+
96	+ +	+	+ -	+ - +	- - - - -	- +	+
97	+ +	+	+ -	- + +	+ - + - -	+ +	+
98	+ +	+	+ -	- + +	- + - - -	+ +	+
99	+ +	+	+ -	- + +	- - - + -	- +	+
100	+ +	+	+ -	- - +	- - + - +	- -	-
101	+ +	+	+ -	- - +	- - + - -	- +	+
102	+ +	+	+ -	- - +	- - + - -	- -	+
*103	+ +	+	+ -	- - +	- - - - -	- -	+
104	+ +	+	+ -	- - -	- - - - -	- -	+
105	+ +	+	+ -	- - -	- - - - -	- -	-
106	+ +	+	- +	+ - -	- + - + -	- -	+

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Pattern Numbers (arbitrary) \ Sites analyzed	1 A§B	2#	3 A#B	4 A B C	13 A§B§C D E	14 A B	15§
143	+ -	-	+ +	- + +	+ + + - -	- +	+
*144	+ -	-	- -	- - -	- - - - -	- -	-
145 (2)	- +	+	+ +	+ + +	+ + + + -	- +	+
146 (2)	- +	+	+ +	+ + +	+ + + - +	+ +	+
147 (2)	- +	+	+ +	+ + +	+ - - - -	+ +	+
148	- +	+	+ +	+ + +	+ - - - -	- +	+
149	- +	+	+ +	+ + +	- + + + +	- +	+
150	- +	+	+ +	+ + +	- + + - +	+ +	+
151	- +	+	+ +	+ + +	- + - - +	- +	+
152 (2)	- +	+	+ +	+ + +	- - + + +	+ +	+
153	- +	+	+ +	+ + +	- - + + -	+ +	+
154	- +	+	+ +	+ + +	- - + + -	- +	+
155	- +	+	+ +	+ + +	- - + - +	+ +	+
156	- +	+	+ +	+ + +	- - + - +	- +	+
157	- +	+	+ +	+ + +	- - + - -	+ +	-
158	- +	+	+ +	+ + +	- - + - -	- -	+
159	- +	+	+ +	+ + +	- - - - -	- +	+
160	- +	+	+ +	+ - +	- - + - -	+ +	+
161	- +	+	+ +	+ - +	- - + - -	- +	-
162	- +	+	+ +	+ - +	- - + - -	- -	+
163	- +	+	+ +	+ - -	+ + + - +	- +	+
164	- +	+	+ +	+ - -	- - + + +	+ +	+
165	- +	+	+ +	- + +	- - + - -	+ -	+
166	- +	+	+ +	- + -	+ - - + -	- +	+
167	- +	+	+ +	- - +	- + - - -	- -	+
168	- +	+	+ +	- - +	- - + + -	- +	+
169	- +	+	+ +	- - +	- - - - -	+ +	+
*170	- +	+	+ +	- - +	- - - - -	- -	+
171	- +	+	+ +	- - -	+ - + + +	+ +	+
172	- +	+	+ +	- - -	- - + - -	+ +	+
173	- +	+	+ +	- - -	- - - + -	- -	-
174	- +	+	+ -	+ - +	- - - + -	+ +	+
175	- +	+	+ -	+ - +	- - - - -	- -	+
176	- +	+	+ -	+ - +	- - - - -	- -	-
177 (2)	- +	+	+ -	- + +	- - - - -	- +	+
178	- +	+	+ -	- + -	- - + - -	- +	+

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Pattern Numbers (arbitrary) \ Sites analyzed	1 A§B	2#	3 A#B	4 A B C	13 A§B§C D E	14 A B	15§
179	- +	+	+ -	- - +	- + + - -	- +	+
180	- +	+	+ -	- - +	- + + - -	- -	+
181	- +	+	+ -	- - +	- + - + -	- -	+
182	- +	+	+ -	- - +	- - + - -	- -	+
183	- +	+	+ -	- - -	- - + + -	- +	+
184	- +	+	+ -	- - -	- - - - -	- +	+
185	- +	+	+ -	- - -	- - - - -	- -	+
186	- +	+	+ -	- - -	- - - - -	- -	-
187	- +	+	- +	+ + -	+ + - + -	+ +	+
188	- +	+	- +	+ - +	- - + - -	- +	+
189	- +	+	- +	- - +	- - - - -	- +	-
190	- +	+	- -	+ - +	- - + + -	- -	+
191	- +	+	- -	- - +	- + - - -	- -	+
192	- +	+	- -	- - +	- - + + -	- +	+
193	- +	+	- -	- - -	- - - - -	- +	+
194	- +	+	- -	- - -	- - - - -	- -	+
195 (3)	- +	+	- -	- - -	- - - - -	- -	-
196	- +	-	+ -	+ + -	- - - - -	- -	+
197	- +	-	- -	- - +	- - - - -	- -	+
*198 (3)	- +	-	- -	- - -	- - - - -	- -	+
199 (2)	- +	-	- -	- - -	- - - - -	- -	-
200	- -	+	+ +	+ + +	- + - - +	+ +	+
201	- -	+	+ +	+ + +	- - + - -	- -	+
202	- -	+	+ +	+ + +	- - - - +	- +	+
203	- -	+	+ +	+ - +	+ + + - -	- +	+
204	- -	+	+ +	+ - +	- - + + +	+ +	+
205	- -	+	+ +	+ - +	- - - - +	- +	+
*206	- -	+	+ +	+ - +	- - - - -	- -	+
207	- -	+	+ +	+ - +	- - - - -	- -	-
208	- -	+	+ +	+ - -	- - - - +	- -	+
209	- -	+	+ +	+ - -	- - - - -	- -	-
210	- -	+	+ +	- + -	- - - - -	+ -	+
211	- -	+	+ +	- + -	- - - - -	- -	+
212	- -	+	+ +	- + -	- - - - -	- -	-
213	- -	+	+ +	- - +	- - + - +	+ +	+
214	- -	+	+ +	- - +	- - + - -	+ -	+

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Pattern Numbers (arbitrary) \ Sites analyzed	1 A§B	2#	3 A#B	4 A B C	13 A§B§C D E	14 A B	15§
215	- -	+	+ +	- - +	- - - - -	+ +	+
216	- -	+	+ +	- - +	- - - - -	- +	-
217 (2)	- -	+	+ +	- - +	- - - - -	- -	+
218 (2)	- -	+	+ +	- - +	- - - - -	- -	-
219	- -	+	+ +	- - -	+ - + - -	- -	+
220	- -	+	+ +	- - -	- - - - -	- -	+
*221	- -	+	+ +	- - -	- - - - -	- -	-
222	- -	+	+ -	+ + +	+ + - - -	- -	+
223	- -	+	+ -	+ - +	- + - - -	- +	+
224	- -	+	+ -	+ - +	- - + + -	+ +	+
225	- -	+	+ -	+ - +	- - + + -	- +	+
226	- -	+	+ -	+ - +	- - - + -	- -	-
227 (2)	- -	+	+ -	+ - -	- - - - -	- -	+
228	- -	+	+ -	- - +	+ + - - -	- +	+
229	- -	+	+ -	- - +	+ - - - -	- -	+
230	- -	+	+ -	- - +	- + - - -	- -	-
231	- -	+	+ -	- - +	- - - + -	- -	+
232	- -	+	+ -	- - +	- - - - -	+ -	-
233	- -	+	+ -	- - +	- - - - -	- +	+
234	- -	+	+ -	- - +	- - - - -	- -	+
235 (2)	- -	+	+ -	- - +	- - - - -	- -	-
236	- -	+	+ -	- - -	+ - + - -	- -	+
237	- -	+	+ -	- - -	- + - - -	- -	+
238	- -	+	+ -	- - -	- - + - -	- -	+
239	- -	+	+ -	- - -	- - - - +	- -	+
240	- -	+	+ -	- - -	- - - - -	+ -	+
241	- -	+	+ -	- - -	- - - - -	- +	+
242	- -	+	+ -	- - -	- - - - -	- +	-
*243 (7)	- -	+	+ -	- - -	- - - - -	- -	+
*244 (13)	- -	+	+ -	- - -	- - - - -	- -	-
245	- -	+	- +	+ + +	+ - - + -	- +	+
246	- -	+	- +	+ - +	- - - - -	- +	+
247	- -	+	- +	- - -	- + - - -	- -	-
248	- -	+	- +	- - -	- - - - -	+ -	-
249	- -	+	- +	- - -	- - - - -	- -	+
*250 (2)	- -	+	- +	- - -	- - - - -	- -	-

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Pattern Numbers (arbitrary) \ Sites analyzed	1 A§	1 B	2#	3 A#	3 B	4 A	4 B	4 C	13 A§	13 B§	13 C	13 D	13 E	14 A	14 B	15§
251	-	-	+	-	-	+	-	+	-	+	-	-	-	-	-	+
252	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
253	-	-	+	-	-	-	+	+	-	-	+	+	-	-	-	+
254	-	-	+	-	-	-	+	+	-	-	-	+	+	+	+	+
255 (2)	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	+
256	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	+
257 (4)	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+
258 (2)	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
259	-	-	+	-	-	-	-	-	-	+	+	-	+	-	-	+
260	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	+
261	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	+
262 (2)	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-
*263 (7)	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
264 (22)	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
265	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+
266	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-
267	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+
268	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-
269	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
*270 (2)	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
271	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+
272	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+
*273	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
274 (2)	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
275	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+
276	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
277 (2)	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
278	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
279	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
*280 (22)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+