MASTER

RADIATION INDUCED DNA SYNTHESIS
IN THE POLYTENE CHROMOSOMES

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Introduction

The study was undertaken to ascertain whether DNA synthesis could be induced in the polytene chromosomes of Drosophila melanogaster by X-irradiation, i.e. whether the production of new breaks in chromosomal DNA would lead to detectably new sites of DNA synthesis. It was hoped that positive results, if obtained, would not only permit an experimental approach to the question of control mechanisms of normal DNA replication but also provide a good basis for exploring the nature of chromosomal breakage and repair in cytologically favorable material. As is detailed below, we have observed induced synthesis of DNA in our material with X-rays as well as with UV light. This synthesis, moreover, can be distinguished from that associated with normal DNA replication.

Materials and Methods

Salivary glands from Drosophila melanogaster (Oregon R) were excised from third instar larvae grown at 18° C in standard corn meal-agar medium, enriched with extra yeast after egg hatching. The larvae were selected among those which had first emerged from the food, and were approaching the prepupal stage, that is about 128 hours after egg laying. At this stage of development, 50% or less of the nuclei from the salivary gland cells, showed no normal label incorporation.

Nuclei from prepupae 2-3 hours after the formation of the "white pupae" were also studied. At this stage, when the

cuticle showed a light brown color, no more than 10% of the nuclei from the salivary gland cells were in DNA synthesis, probably the last replication before pupation.

We have also used glands which were transplanted (from synchronously grown third instar larvae) into the abdominal cavity of adults and cultured for nine to nineteen days. Two mutants which were considered of interest in this context were also included. They are the cross-over suppressor C(3)G and a developmental mutant, lethal giant larvae l(2)gl.

The two lobes from each gland were separated and one used for treatment, the other for control. They were placed individually in the depressions of a disposable microtiter plate containing a small amount of Ephrussi-Beadle medium (Ephrussi and Beadle, 1936).

The experimental gland halves were irradiated and high specific activity tritiated thymidine (12 c/mM) was subsequently added to both experimental and control gland halves. A solution of ten microcuries per milliliter of tritiated thymidine was employed for a 15 minute incubation.

Several doses of X-rays (1000r, 5000r, 10,000r and 30,000r) were used in the first experiments. Later 100,000 was routinely used. After incubation in thymidine, the glands were smeared on gelatinized slides in a drop of 45% acetic acid fixation. After removal of the cover slip, they were post-fixed in 1:3 acetic-alcohol, stained in a solution of 2% orcein in 65% acetic acid for 15-20 minutes, dehydrated, passed through a solution of 1% parlodion in amyl acetate, run down to distilled

water, and finally covered with Kodak A.R. 10 autoradiographic stripping film or dipped in Ilford K5 nuclear research emulsion mixed with deionized water in 1:1 w/v at 45°C.

The slides thus prepared were placed in light-proof boxes and stored at 4°C for two weeks after which they were developed in D19B Kodak developer and examined.

For the irradiations with ultraviolet light a GE germicidal source was used. A total dose of $1800 \text{ ergs/mm}^2/\text{sec}$, was usually delivered to the glands which were flattened without breaking the cells in a drop of Ringer with quartz cover slips and subsequently incubated. Even under these conditions a total, even penetration was not obtained.

The specificity of thymidine labeling for DNA was checked through the use of DNase on some experimental as well as control slides prior to autoradiography (Worthington pancreatic DNase, 30 mg/100 ml in $4 \times 10^{-3} \text{ MMgSO}_4$ at pH 7.0, 38°C, 3-4 hours). No radioactivity was observed in any of the slides so treated.

Analytical Procedure

Several hundred experimental and control nuclei were analyzed following the procedure outlined below. The parameters taken into consideration were as follows:

1. Frequency of labeled nuclei.
2. Proportion of "discontinuous" and "continuous" labeling.
3. Frequency of labeling at specific loci and frequency of joint labeling at normally correlated loci.
4. Iso-labeling at homologous loci in unpaired regions of homologous chromosomes.

5. Distribution of autoradiographic grains across versus along the chromosome axis.

It has previously been observed in this laboratory that during normal chromosomal DNA replication, labeling is always present in the chromocenter. Chromocentral labeling was therefore used as the criterion for determining, especially in doubtful cases, whether or not a nucleus was undergoing normal DNA replication.

A nucleus was considered continuously labeled when the label was distributed along the chromosomes without obvious interruption. In cases of light spot labeling along the chromosomes a cluster of at least 4 grains referable to a band or small chromosome region was taken as indication of localized DNA synthesis.

When considering the pattern of labeling at individual and correlated loci only those chromosome segments where the patterns of incorporation had already been established (Howard & Plaut, 1968) were observed. These segments are (according to Bridges' salivary chromosome map) regions 1, 2, 3, and part of 4 of the X chromosome; regions 58, 59, and 60 of chromosome II R and 61, 62, 63, and part of 64 of chromosome III L.

Results

Experiments using freshly excised salivary glands from late 3rd instar larvae and light brown pupae.

It has been possible to establish that X-irradiation leads to synthesis of DNA at a large number of sites on the chromosomes as manifested by the incorporation of H^3 -thymidine. The sites of

incorporation appear randomly scattered over the euchromatin; few or no silver grains have been detected over the chromocentral region. We suggest that this process represents the repair of X-ray damage to chromosomal DNA and that it resembles the phenomenon of repair replication described in bacteria and other cells. We have observed such induced DNA synthesis with all X-ray doses used except the lowest, 1000r.

The identification of labeling as induced is based on the following considerations: in untreated glands, about 50% or less of the nuclei show label incorporation. Only a few of these nuclei have continuously labeled chromosomes; the majority are spot labeled with recognizable and predictable patterns. In glands irradiated with doses above 1000r the frequency of labeled nuclei greatly exceeds 50% (up to 95% with a dose of 100,000R). (See table 1). Moreover, a number of the labeled nuclei in irradiated glands had unlabeled chromocenters and were thus not undergoing normal replicative DNA synthesis. These nuclei showed random labeling over the euchromatic chromosome regions and no tendency toward spot labeling patterns. Since this particular distribution of incorporation sites is never seen in unirradiated nuclei its identification as induced is definite.

Experiments with glands cultured as implants:

Dr. and Mrs. Cordeiro, in our laboratory, had discovered that 95% or more of salivary gland nuclei (from synchronized mid-third instar larvae) cultured as implants in the abdominal cavity of adult females would stop incorporating tritiated thymidine on about the 7th or 8th day after implantation.

(These nuclei, even when aged for almost thirty days in implants, retain the ability to incorporate tritiated uridine and are thus viable). The fact that normal chromosome replication was suppressed (or terminated) in almost all of these nuclei led us to inquire whether cultured nuclei were responsive to the action of radiation and whether it might therefore be possible to study induced synthesis in the absence of normal replication.

It was found that implanted glands do indeed show an increase in labeling following irradiation in a manner quite comparable to freshly excised glands. In a typical experiment nuclei were irradiated with 30,000r 8, 15, and 19 days after implantation and immediately incubated with tritiated thymidine; an average of 50% showed induced incorporation of the label, although this was sparse in comparison with that observed in freshly excised glands. About 2% of the experimental and control nuclei evidenced normal replication in the form of continuous or spot labeling. Experiments using different doses such as 5000r and 15,000 r gave generally similar results.

Experiments with mutants

Since the phenomenon of repair replication has been shown to be under genetic control, the study of certain variations may afford important information. With this idea in mind we are studying and selecting mutants of D. melanogaster whose phenotypes would lead us to suspect variations in DNA and RNA synthesis at various times during development.

We have selected so far some obvious ones such as lethal giant larvae (which affects development) and the crossover

suppressor C(3)G (affecting crossing over).

Lethal giant larvae, l(2)Gl, is a mutant which is located in the second chromosome at 0.0. It was selected because it shows a number of developmental abnormalities correlated with a reduced amount of DNA in the nuclei of the salivary gland, being only 20% of that of the normal 3rd instar, or equivalent to an 80 hour wild type larvae in development and DNA content. Also the amino acids, peptides and proteins, as well as the hormone level, are affected (Lindsley and Grell, 1967).

We have determined that late 3rd instar larvae of this mutant do not incorporate H^3 -thymidine in at least 80% of their salivary gland nuclei, with the rest showing mostly a continuous but light pattern of incorporation. Labeling with H^3 -uridine is also affected, appearing light and spotty in comparison with the wild type, suggesting that transcription may be affected. When glands of such larvae were examined for induced DNA synthesis after X-irradiation, we observed a distinct but light incorporation of the label in 50-60% of the nuclei. There seems, however, to exist a decrease in response with approaching death at pupation.

The other mutant, C(3)G, located in the 3rd chromosome at 57.4 results in almost no crossing-over in the entire genome in homozygous females (Lindsley and Grell, 1967). From the homozygous stock ($\frac{\text{ve h th C(3)G-Sb Ubx}}{\text{st C(3)G-ca}}$) cultured at room temperature, late 3rd instar larvae were dissected and irradiated. Autoradiographs showed that, as in the wild type, unscheduled incorporation was stimulated and to a similar extent.

Table I, X ray-induced synthesis in the nuclei of salivary gland cells.

Type	Exposure	No. of cells scored	% unlabeled	% labeled
Late 3rd instar larvae	0 KR	1000	40	60
	100 KR	1000	5	95
Light brown pupae	0 KR	1000	90	10
	100 KR	1000	35	65
3rd instar implants	0 KR	~50	95	<5
	30 KR	~50	50	50
Lethal giant larvae. Late 3rd instar	0 KR	1000	95	5
	100 KR	1000	40	60

Note - The cells studied in the transplants and l(2)gl showed a noticeably lower frequency of grains induced by radiation in comparison with those from 3rd instar or light brown pupae.

Table 2. Average number of grains per nucleus. (At least 500 nuclei were scored and the background was subtracted after counting the grains from an equivalent area for each nucleus.

Dose Class	5KR	30KR	100KR
Oregon R 3rd Instar	45	100	200
Oregon R light brown pupae	--	--	40
transplanted nuclei from 3rd instar Oregon R	less than 10	10	
lethal giant larval 3rd instar (158 h. old)	--	--	50
c3G homozygous females 3rd instar	--	--	200

Discussion

The results obtained show clearly that a range of doses of X-rays and UV light induce a kind of DNA synthesis in the salivary gland nuclei which can be detected by the incorporation of tritiated thymidine and which appears to be closely related to, if not identical with, the phenomenon of repair replication known in bacteria (Setlow and Carrier, 1964; Boyce and Flander, 1964) and reported for mammalian cells (Rasmussen and Painter, 1966; Painter and Cleaver, 1968).

In our system this effect has been induced and observed in the extended interphase polytene chromosomes at highly localized single spots, which are randomly distributed throughout the euchromatic chromosome region.

No autoradiographic grain clusters were observed at any points along the chromosomes and there was no evidence of iso-labeling in unpaired regions, indicating that the events must be highly localized, probably not affecting more than part of a single chromosome (or single DNA helix) in the closely packed bundle. It would appear that this generalized and yet local effect of radiations is not equivalent to the stimulus responsible for normal chromosomal DNA replication because if it were, one should observe clusters characteristic of the temporal patterned replication (Plaut, Nash, Fanning, 1966; Howard and Plaut, 1967).

Thus the incorporation of the label is probably not a partial reinitiation of normal synthesis, but a separate process which can operate independently. This conclusion is also supported by

the fact that in the transplanted glands as well as in the brown pupae and in late 3rd instar of l(2)gl induced synthesis occurs in nuclei which were no longer engaged in normal replicative DNA synthesis. In replicating nuclei, both processes may occur simultaneously.

It is evident from tables 1 and 2 that the frequency of labeled nuclei and the average number of grains detected in the brown pupae and in the 3rd instar larvae of lethal giant larvae, are considerably less than in 3rd instar wild type. In the brown pupae it would appear that the cessation of the replicative ability is related to the process of metamorphosis and this is in time related to a considerable diminution in the effectiveness of the repair system.

In lethal giant larvae the lower frequency of labeled nuclei and lower grain counts are probably correlated also with a marked paucity in replicative activity which might be a manifestation of the mutant gene resulting in a diminished polyteny (20% of that of the normal 3rd instar). It is note-worthy that those nuclei which incorporated the label showed a repair capacity (or activity) greater than that of the light brown pupae with a larger DNA content but already undergoing a profound change in differentiation (metamorphosis). From these observations it appears that the regulation of this inducible synthesis activity is influenced by but not fully regulated by the changes brought about by differentiation (and/or hormone level) as seems to be the case in normal replication of DNA. We can also see that the mutant gene, l.g.l., which may well be

affecting (controlling) the rate of DNA synthesis and of development of the fly during larval stages, does not prevent unscheduled synthesis, rather, it would appear to maintain a level proportional to the ploidy (or DNA amount).

The transplanted salivary gland nuclei probably continue normal replication for about one more cycle at the most in only 5% of the nuclei, usually during the first 8 days but it could be prolonged for as much as 15 days. This prolongation of the life of the gland up to 18 days results in a progressive degeneration of the nuclei and extremely enlarged chromosomes. Yet at this point we were able to detect a weak response to radiation, indicating that the system which controls inducible synthesis is still operative. These results point in the same direction as those mentioned above. The repair system may operate independently and under different physiological conditions than the replicative system.

Assuming that the number of grains observed (table 2) represents the label incorporated during unscheduled synthesis at that many single strand breaks, it is of interest to make an evaluation of the efficiency of our technique.

According to Veatch (1969) the number of single strand breaks which are induced in general varies from 0.2 to 3 per rad per 10^{12} daltons. Adapting an estimate for *Drosophila* of 1 break per rad, the polytene nuclei with 7×10^{13} daltons or would yield well over 10^6 single strand breaks at 100 KR. That is, at least 1 single strand break per 10^7 daltons or 200-300 single strand breaks per band or 14×10^7 daltons.

Lett (1967) has found that single strand breaks rejoin at the rate of 50% in the first 30 minutes. Since we labeled for only 15 minutes after irradiation we are detecting at most about 1 single strand break per 1000 induced.

All heterochromatic regions comprised within the chromocenter are characterized by the dearth of induced label incorporation. One is tempted to relate this observation to the so-called "breakability" shown by the *Drosophila* chromosomes from the germinal line in genetic experiments. It is probably true that DNA in heterochromatin by itself is not more breakable than that of euchromatin but, being complexed with proteins in a more condensed state, the binding sites induced by radiation may not be readily recognized by the polymerase and/or the necessary strand degradation of the broken ends prior to repair may require longer time than in the less condensed euchromatic regions. Then unscheduled synthesis to repair these lesions would proceed at a slower rate (as may also be the case for normal replicative synthesis). The grain yield would thus be lower and possibly not detected with the short pulses used.

In the case of the mutant c3G the suppression of recombination in the homozygous female does not seem to be correlated with a deficiency in unscheduled synthesis as has been observed in *rec⁻* bacteria and its response to radiation does not differ from that observed in the wild type.

The suppression of crossing over in c3G may be due then to some other reason, perhaps, as has been postulated (Lindsley et al., 1968), it affects the process of pairing before recom-

bination takes place. Or, considering that mitotic recombination is not affected in homozygous females (Le Clerc, 1946), it may be that the mutant effect is tissue specific and the suppression of recombination, if it exists, can only be detected in cells from the germinal line.

Watson (1968) concluded that repair was absent in c3G individuals as he did not observe incorporation of tritiated thymidine in the salivary gland nuclei; on this premise Green (1970) inferred that his recently discovered mutator, mt, apparently an allele of c3G may be interfering with or suppressing repair (or un-scheduled synthesis). Though this interpretation appears unlikely in the light of our present results with c3G, it would be of interest to test further this mutator allele.

The experiments discussed above were repeated using UV light and the results obtained showed that the presumptive lesions induced stimulate the incorporation of tritiated thymidine, raising the frequency of labeled nuclei up to 80% in some cases. The distributional pattern of the grains is indistinguishable from that found for X-rays. UV light induces its damage mainly through the formation of thymidine dimers which are excised in the form of small oligonucleotides. These in turn are replaced by new intact nucleotides covalently joined at the ends to the repaired strand by ligase action. This process of repair is best known in E. coli and apparently occurs in human cells as well (Regan et al. 1968). However, no dimer excision has yet been found in other mammalian cells (Trosko et al. 1964). It is not known whether or not dimers are formed in the polytene

chromosomes. However, Muller (1954) has reported that polar cap cells of the *Drosophila* embryo, irradiated with low doses of UV and exposed to photoreactivation conditions afterwards show a reduction of 75% in the total mutational damage. This observation argues in favor of the possibility that dimers may be formed and that they may be monomerized by a photoreactivating enzyme in the polytene chromosomes.

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