Deoxyribonucleic Acid Synthesis in Relation to Duplication of Centers in Dividing Eggs of the Sea Urchin, *Strongylocentrotus purpuratus**

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ABSTRACT

The earliest known event in the sequence leading to mitosis is the duplication of cell centers. The present investigation shows that the synthesis of DNA, although closely following it in time, is initiated entirely independently of this prior event. Fertilized eggs of the sea urchin, *S. purpuratus*, were exposed to β-mercaptoethanol at intervals during development. This substance, when introduced at appropriate times, blocks mitosis and also prevents duplication of centers. Whether or not duplication of centers had already occurred before introduction of the blocking agent was determined by observing the division patterns of eggs after the mercaptoethanol was removed: division of one cell into two, or of two into four indicated that duplication had not occurred; division of one into four or of two into eight, that it had. Incorporation of H^3-labeled thymidine into DNA, as demonstrated by autoradiography, showed that DNA synthesis took place during the mercaptoethanol block regardless of whether or not the centers had already duplicated. Thus the two major reproductive events of the mitotic sequence, although normally coordinated in time, can be dissociated experimentally and shown to function independently.

INTRODUCTION

The mitotic distribution of genetic material to the progeny of a dividing cell is preceded by a number of preliminary events, commonly described as preparations for division. These preparations include the doubling of chromosome substance, the reproduction of the centers, the assembly of the mitotic apparatus, and the allocation of energy specifically for division (2).

At the present state of our knowledge, the earliest clearly definable event in the mitotic sequence is the duplication of the cell centers (or centrioles) which, in the sea urchin at least, occurs during the final stages of the division preceding the one in which they are to serve as separate functioning units (2, 5). The question arises as to whether this might serve as the initiating event in an interdependent series, or whether each requisite process occurs independently in parallel, and not until all are completed can the coordinating mitotic machinery go into operation.

At first glance, this question might seem to have been answered by the numerous older cytological observations of the independence of chromosome reproduction from the reproduction of the centers. The most familiar class of such cases is endomitosis and related forms of endoreproduction such as polyploidization by colchicine and other agents. Here the chromosome complement increases without a visible increase in the number of centers. However, it has now been shown that the fundamental reproduction of the centrioles and the division of the progeny to form distinguishable mitotic centers are separable processes (5). The centers are normally double units, can separate into single units under experimental condi-

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tions, and it is entirely conceivable that they may in cases in which endoreproduction of the chromosomes takes place, also reproduce without division to form polyploid centers. The purpose of the present study was to test this possibility.

It is possible to determine whether or not the centers have duplicated through the use of β-mercaptoethanol. Two effects of mercaptoethanol have been described: (*a*) it inhibits mitosis if applied at any time prior to metaphase, and (*b*) it inhibits the duplication of centers. However, it does not interfere with the splitting of centers, nor does it prevent their separation to opposite nuclear poles at appropriate times in the mitotic sequence (3, 6, 5). These findings permit the designing of experiments to find out whether the synthesis of deoxyribonucleic acid (DNA), which follows closely upon the replication of the centers, is or is not dependent upon the prior occurrence of this initial event in the mitotic sequence.

The following additional information, which was available to us from prior work of T. Bibring,[1] was essential to the plan of the present experiments. (*a*) The sea urchin eggs take up thymidine rapidly. (*b*) Mercaptoethanol *per se* does not block the incorporation of thymidine into DNA. (*c*) If the cells are blocked by mercaptoethanol at metaphase, they do not incorporate thymidine into chromosomal DNA as long as the block continues. It is important to note that points (*b*) and (*c*) are not contradictory; they say that mercaptoethanol does not inhibit DNA synthesis directly, but can do so indirectly by keeping the chromosomes in their condensed mitotic form. This is in agreement with all previous information (9) to the effect that chromosomes do not synthesize DNA during mitosis.

The experimental design is as follows: mercaptoethanol is added to a suspension of fertilized eggs either before or after the centers have duplicated. A short time later tritium-labeled thymidine is added, and incubation is continued sufficiently long to permit the splitting and separation of whatever centers are present. The appearance of radioactivity in the nuclei during this time serves as an indication of whether or not DNA synthesis has occurred during the mercaptoethanol block. The cells are then washed free of mercaptoethanol and their pattern of division observed during recovery from the block. If duplication of centers has not taken place, one cell will divide into two; if it has, one cell will divide directly into three or four daughter cells. The evidence justifying this criterion for duplication of centers has previously been reported by Mazia *et al.* (5).

[1] Thomas Bibring, Doctoral Thesis, Department of Zoology, University of California, Berkeley, in preparation.

Materials and Methods

All experiments were carried out with *S. purpuratus*, collected in the San Francisco region. Eggs were suspended in sea water, fertilized, incubated at 15°C., and treated with mercaptoethanol as previously described (5). β-Mercaptoethanol, obtained from Eastman Organic Chemicals Department, Rochester, N.Y., was employed in a final concentration of 0.075 M.

Incorporation of tritium-labeled thymidine into DNA, as determined by autoradiography, was used as an index of DNA synthesis. Thymidine of high specific activity (3.0 curies per mM), obtained from Schwarz Laboratories, Inc., Mt. Vernon, New York, was added to produce final concentrations of 3 or 10 microcuries per ml. of egg suspension.

At specified intervals eggs were fixed in alcohol–acetic acid (3:1), embedded in paraffin or methacrylate (8 parts butyl to 1 part methyl), and sectioned at 3 or 4 microns respectively.

Slides were stained by the Feulgen method. Plates of autoradiographic stripping film were immersed briefly in 100 per cent ethanol, then transferred to a dish of 95 per cent ethanol in which the film was scored with a razor blade and gently peeled off the plate. It was floated on water, and applied to the slides in the usual manner. The alcohol stripping technique was found helpful in avoiding the flashing due to static electricity that commonly occurs in dry climates (4).

RESULTS

First Division.—Eggs from a single female were fertilized at zero time, subdivided into 1 ml. batches and incubated at 15°C. At the times shown in Table I mercaptoethanol was added, followed 10 or 20 minutes later by labeled thymidine. The delayed addition of the thymidine allowed time for the mercaptoethanol block to become established. After approximately 90 minutes in mercaptoethanol, samples from each batch were fixed in alcohol–acetic acid. This was the point at which untreated controls were passing through the two-cell stage. The remainder of each batch was continued in the blocking medium for a total of 3 hours. By this time the centers in the blocked eggs had ample time to split and separate, since untreated controls were now passing through the

TABLE I

Effects of Mercaptoethanol on Incorporation of H^3-Thymidine into DNA and on Duplication of Centers during First Cleavage

Added ME* at min.	Added H^3-thymidine at min.	Stage at introduction of ME	Stage after 90 min. in ME	H^3 in nuclei after 90 min. in ME	Type of cell division during recovery after 180 min. in ME	State of centers
15	25	Two pro-nuclei in all cells	Two pro-nuclei in nearly all cells	All nuclei labeled whether fused or not	Approximately half divided 1 → 2, and half 1 → 3 or 4	Approximately half duplicated and half not
20	30	Few still unfused; most show fusion nuclei	Most nuclei fused; some in prophase; few in metaphase	All nuclei labeled regardless of phase	Most cells divided 1 → 3 or 4	Most duplicated
25	45	Fusion nuclei	Few in prophase; most in metaphase	Prophases labeled as above; metaphases very lightly labeled	No 1 → 2 divisions seen; all dividing 1 → 3 or 4	All duplicated
60	80	Most in prophase	90 per cent blocked in metaphase; rest passed block and divided to two cells.	A few nuclei labeled, probably from two-cell stage, but *no* metaphases labeled	Divided 1 → 3 or 4 and 2 → 4; a few progressed to higher cell numbers; asynchronism	All duplicated for first division; for second division most not

* ME is mercaptoethanol.

eight-cell stage; accordingly the mercaptoethanol was removed so that the pattern of cleavage could be observed during the recovery period.

Development of eggs exposed to mercaptoethanol is not blocked unequivocally at the outset; rather, as with many other mitotic inhibitors, the stage of final arrest varies depending upon the stage at which the blocking agent is introduced. As shown in Table I, when mercaptoethanol was added 15 minutes after fertilization, the egg and sperm nuclei failed to fuse and persisted as separate entities as long as the blocking agent was present. During this time, however, DNA synthesis occurred, since all nuclei were labeled. When allowed to recover, approximately half of the cells divided from one cell into two, and the remainder from one into three or four, an indication that in around 50 per cent of the cases the centers had not yet duplicated. (A division of one to three, in addition to indicating polyspermy, may mean that only one unit of the normally duplex center has completed its duplication, or that, although two duplex centers have formed, one had either failed to split or its halves failed to migrate; instead of a tetrapolar a tripolar spindle results.) Since no unlabeled nuclei were found, it appeared that DNA synthesis was initiated independently and did not depend on the prior duplication of the centers.

When mercaptoethanol was added at 20 minutes after fertilization some progression occurred during the block in eggs with fused nuclei, so that some prophases and even a few metaphases were observed. Thymidine was added 10 minutes after the mercaptoethanol and labeling was present in all instances. The recovery pattern showed that by this time the centers had duplicated.

In the following batch mercaptoethanol was introduced at 25 minutes and labeled thymidine 20 minutes later. Most cells progressed to metaphase during the block, and these metaphase figures were only very lightly labeled, presumably because DNA synthesis was nearly complete by the time the labeled precursor was added. Persistence of the metaphase condition due to the mercaptoethanol apparently prevented further DNA synthesis.[1]

This finding was further borne out by the batch of eggs blocked at 60 minutes with introduction of the H^3-thymidine at 80 minutes. By this time 90 per cent of the cells proceeded to metaphase and were held there, while the remainder, having already passed this critical point, were able to divide into two cells. As a result, a few nuclei, derived from the latter cells, were labeled, but none of the metaphase figures showed any labeling.

Second Division.—The above experiment was repeated with emphasis on the second division instead of the first, since the first mitosis of the fertilized egg is somewhat atypical, particularly as regards the origin of the centers. The strategy in this case depends on the following observations: (*a*) introduction of mercaptoethanol before or after the *first metaphase* determines whether or

TABLE II

Effects of Mercapoethanol on Incorporation of H^3-Thymidine into DNA and on Duplication of Centers during Second Cleavage

Added ME at min.	Added H^3-thymidine at min.	Stage at introduction of ME	Stage after 200 min. in ME	H^3 in nuclei after:			Type of cell division during recovery from ME	Stage of centers
				110 min. in ME	200 min. in ME	Recovery from ME		
60	80	1st prophase	Blocked, in metaphase	None	None	All labeled	Most cells divided 1 → 3 or 4	Duplicated for first division
70	90	1st prophase and approaching metaphase	Blocked, in metaphase	None	None	All labeled	Most cells divided 1 → 3 or 4	Duplicated for first division
80	100	Approaching first metaphase	Most in metaphase. Few have divided to two	Metaphases not labeled; nuclei from two cells densely labeled		All labeled	Few 1 → 3 or 4; mostly 2 → 3 or 4	Second duplication blocked
90	110	Most in first metaphase, some in anaphase	80 per cent in metaphase. Rest two-cells with micronuclei and persisting asters	Metaphases not labeled; nuclei from 2 cells densely labeled		All labeled	Few 1 → 3 or 4; and a few 2 → 6 or 8	Mostly blocked; few passed block
100	120	First anaphase and telophase	20 per cent in metaphase. Rest two-cells with micronuclei	Metaphases not labeled; nuclei from 2 cells densely labeled		All labeled	Some 2 → 4, and many 2 → 6, 7 or 8	Some blocked and some duplicated
110	130	Most beginning first division	Over 90 per cent two-cells nearly all in second metaphase.	Rare unlabeled first metaphase. Others all labeled		All labeled	Mostly 2 → 6, 7, or 8	Most duplicated

not the cells will remain blocked or divide into two, and (*b*) during this time the centers are duplicating in preparation for the second mitosis, at the start of which four duplex centers are normally present. Whether or not duplication of centers has occurred is indicated by whether the cells recovering from the block divide from two to four or from two to eight respectively.

The experimental plan and the results are shown in Table II. The earliest exposure to mercaptoethanol (60 minutes) was before the first metaphase, and the latest (110 minutes) was just as the cells were beginning to divide. A 20 minute interval was allowed throughout for the mercaptoethanol block to become effective before introduction of the H^3-thymidine. Samples of cells were examined after both 110 minutes and 200 minutes in mercaptoethanol. The results confirmed the previous findings: all nuclei were labeled except for those which were nearing metaphase and completion of DNA synthesis before exposure to the H^3-thymidine. As before, the initiation of DNA synthesis did not hinge upon duplication of the centers, since the labeling was the same even when center duplication was suppressed, as shown by recovery patterns of two cells dividing to four.

At the end of 110 minutes' exposure to mercaptoethanol, at the time the first set of samples was taken, the remaining cells were washed three times with a solution of non-labeled thymidine and mercaptoethanol in sea water, in order to remove the isotope or dilute it to ineffectiveness with unlabeled material and thus stop further incorporation. (The non-labeled thymidine was 100-fold more concentrated than the isotopically labeled compound.) An additional 90 minutes' incubation was carried out in mercaptoethanol plus non-labeled thymidine with the non-labeled compound. Samples taken at the end of this time (200 minutes in mercaptoethanol) showed no labeling of metaphases. However, even when the mercaptoethanol and any residual extracellular H^3-thymidine were removed by three additional washings with non-labeled thymidine solution and the cells allowed to recover, all nuclei were heavily labeled. It appears that even though DNA synthesis could not proceed in cells with condensed chromosomes, thymidine was being taken up and possibly incorporated into DNA precursors, so that removal of the block resulted in early nuclear labeling in these cells.

These precursors are obviously some inter-

mediates on the pathway between thymidine and macromolecular DNA, and the fact that they were not detected in autoradiographs suggests that they are low molecular weight units, soluble in the alcohol-acetic fixative or in the solutions used to prepare the eggs for autoradiography. A likely guess is that they are di- or triphosphates of thymidine.

The failure of DNA synthesis to occur during metaphase was clearly not due to any inhibitory effect of mercaptoethanol upon this process, but was secondary to the prolongation of chromosomal condensation by the blocking agent.

DISCUSSION

A number of distinct mechanisms are now recognized as capable of exerting a measure of control over normal cell division (10, 2). Their interrelations are obscure, although it is obvious that some sort of machinery must exist to integrate them into a coordinated process. In the present study a search was made for a possible interaction between two of these mechanisms. Since certain inhibitors, for example ultraviolet radiation, when applied beyond a certain point, affect not the current division but the subsequent one (1), we focused our attention upon the first identified event of a given mitosis—the duplication of the centers. That this process might serve to pace later events in the time sequence is an extremely attractive hypothesis, but our results clearly show that it is untenable; the synthesis of DNA, which is obviously of supreme importance to the mitotic function, is initiated at the appropriate times regardless of whether replication of centers has or has not occurred.

It is interesting in this regard that in the first division in the sea urchin, although DNA formation is normally preceded by pronuclear fusion, it proceeds at the proper time in mercaptoethanol-treated eggs, even though the two nuclei remain unfused. When cells are blocked at metaphase and the chromosomes are held in the condensed state further synthesis does not occur, but it resumes promptly by the end of telophase or early interphase. The evidence suggests that the formation of DNA precursors may go on even during the metaphase block. This rapid resumption of synthesis at the end of telophase is characteristic of normal non-blocked eggs also (7, 8). DNA synthesis follows the nuclear cycle, even if the extranuclear mitotic events are distorted.

Although mercaptoethanol prevents duplication of the cell centers they are able to separate and migrate at the usual intervals, an indication that, like DNA synthesis, these latter processes are functioning normally. If there is a single initiating mechanism for mitosis and if it should reside in the centers it must be independent of their prior duplication. Only by exploring such relations stepwise can a final picture be drawn.

SUMMARY

When the first or second divisions of fertilized eggs of *S. purpuratus* were blocked by mercaptoethanol, H^3-labeled thymidine was incorporated into DNA regardless of whether or not duplication of the cell centers had occurred. Thus the initiation of DNA synthesis appears to be independent of the mechanism which produces focal centers for the organization of the mitotic apparatus.

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