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*CHANGES IN THE DISTRIBUTION OF POLYMERASE ACTIVITY DURING DNA SYNTHESIS IN MOUSE FIBROBLASTS**

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The availability of partially synchronized cultures of mammalian cell lines[1, 2] provides a new opportunity to investigate the control of DNA synthesis in the cells of higher organisms, which should supplement the extensive studies with lower forms.[3] In this paper will be described the extent and timing of DNA synthesis in mouse fibroblasts (L cells) partially synchronized with 5-fluorodeoxyuridine[4] as well as changes in the intracellular distribution of DNA polymerase activity which suggest that the particulate polymerase may be of more importance than recognized previously. During the course of this work, we learned that results similar to ours have been obtained by Gold and Helleiner.[5] Recently, Billen has described a polymerase-DNA complex in *E. coli*.[6]

Experimental.—L cells were grown in suspension as described previously.[4] Cell counts were obtained with the Coulter electronic counter model A; after preliminary studies an aperture current setting of 3 and threshold of 20 were used routinely. Such cell counts averaged 6 per cent greater than simultaneous chamber counts.[7]

The rate of incorporation of hypoxanthine-8-C^{14} (California Corporation for Biochemical Research) into DNA was determined by incubation of duplicate 1 ml aliquots of the suspension culture with a saturating concentration of hypoxanthine-8-C^{14} (3.7×10^{-5} *M*; 7.6 μc per μM) for 1 hr at 37°C in a Dubnoff shaker. Then the cells were diluted with 4 ml of cold 0.14 *M* NaCl solution and collected by centrifugation; the acid-soluble material was extracted with cold 5 per cent trichloroacetic acid and the residue digested with *N* NaOH for 16 hr at 37°C to hydrolyze RNA. After cooling, DNA and protein were precipitated with acid, collected by filtration on a cellulose filter (Millipore Filter Corporation; type AA filter with pore size 0.8 μ), washed four times by filtration of 5 ml of cold 5 per cent trichloroacetic acid, and assayed for radioactivity as described previously for thymidine-2-C^{14}.[4] All samples were counted to less than 5 per cent error. Essentially all incorporated radioactivity became acid-soluble if the cells were treated with 5 per cent trichloroacetic acid at 90°C for 20 min.

For the usual assay of DNA polymerase activity, 3×10^6 cells were collected by centrifugation, suspended by agitation in 1 ml of cold buffer containing 0.1 *M* KCl and 0.02 *M* tris(hydroxymethyl)aminomethane (pH 7.4), and transferred to a small cellulose test tube. After centrifugation at 4°C, the cells were suspended in 100 μl of cold 0.02 *M* tris(hydroxymethyl)aminomethane (pH 7.4), disrupted by vibration at 10 kc per sec for 2 min in a Raytheon Model DF 101 sonic oscillator, and separated into a supernatant fraction and a pellet by centrifugation at about $6{,}000 \times g$ for 30 min at 4°C. In this paper, the pellet will be referred to as the particulate fraction, although it is recognized that its components have been altered by sonic oscillation. The particulate fraction was washed once with cold 0.02 *M* tris(hydroxymethyl)aminomethane and suspended in 50 μl of the same buffer. The concentration of protein in each fraction was deter-

mined.[8] The incubation mixture (total volume 25 μl) contained: 10 μl of either fraction (25 to 75 μg of protein), as well as ATP 5×10^{-3} *M*; d-ATP, d-CTP, and TTP, each 1.35×10^{-4} *M*; d-GTP-8-C^{14} (3 μc per μM), 4×10^{-5} *M*; $MgCl_2$, 5×10^{-3} *M*; tris(hydroxymethyl)aminomethane, 2×10^{-2} *M* (pH 7.4); and 200 μg per ml of DNA (Worthington Biochemical Corporation) which had been heated at 100°C for 5 min and rapidly cooled. After incubation for 1 hr at 37°C, 5 ml of cold 5 per cent trichloroacetic acid were added to the incubation mixture, and the resulting precipitate was collected on a cellulose filter, washed with cold acid four times, and assayed for radioactivity as described above. 0.1 μg of C^{14}-DNA could be quantitatively recovered on the filter under these conditions, even in the absence of protein.

Essentially no radioactivity was incorporated if the incubation mixture containing the supernatant fraction was held at 0°C or if DNA or enzyme was omitted. A higher concentration of DNA, ATP, or the deoxyriboside triphosphates did not increase the incorporation, nor was it increased by the addition of albumin, KCl, mercaptoethanol, or ethylene-diamine tetraacetate to the cells before disruption or to the incubation mixture. The rate of incorporation was constant for at least 60 min. Over the range of protein concentration used, incorporation was proportional to protein concentration. Polymerase activity did not decline if cells were kept in culture medium at 4°C for 24 hr prior to disruption; the activity of cell-free extracts stored at 0 or −20°C declined after 24 hr. The polymerase activities in Figure 3 and Table 1 represent the average of determinations on fractions from duplicate aliquots of cells.

d-GTP-8-C^{14} was synthesized enzymatically from d-GMP-8-C^{14} (Schwarz BioResearch, Inc.)[9] and separated from the other components of the reaction mixture by paper electrophoresis at pH 3.0.

Results.—Extent and timing of DNA synthesis: The increases in the number of cells and in the amount of DNA per ml during partially synchronized growth of L cells in suspension culture are shown in Figure 1. In three such experiments, approximately 15 per cent of the inoculated cells divided during the first 15 or 16 hr after addition of 5-fluorodeoxyuridine, and the remaining 85 per cent divided after release with thymidine at 15 or 16 hr, mainly between 24 and 32 hr. Other experiments have indicated that delay beyond 16 hr before adding thymidine leads to decreased cell viability, as reported previously for HeLa cells[2, 10] and that concentrations of 5-fluorodeoxyuridine or thymidine other than those used in the experiment shown in Figure 1 or the inclusion of deoxycytidine (2×10^{-5} *M*)[11] did not improve the degree of synchrony.

In three experiments exemplified by that shown in Figure 1, the content of DNA per cell averaged 15.4 $\mu\mu$g at the time of addition of 5-fluorodeoxyuridine, 14.8 $\mu\mu$g when thymidine was added, and 20.5 $\mu\mu$g just before cell division. If the amount of DNA present during this division (e.g., at 27 hr in Fig. 1) was regarded as the basal amount for the number of cells present after division (e.g., at 37.5 hr in Fig. 1), the basal amount of DNA per cell

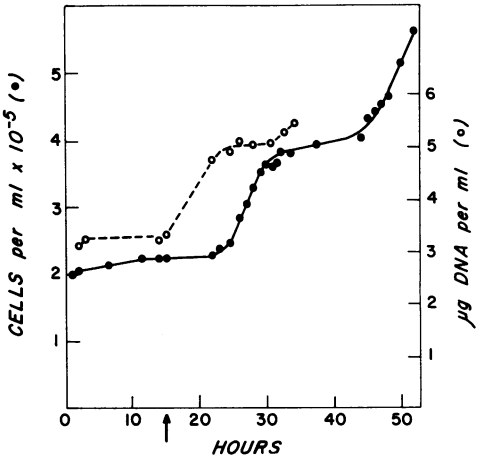

FIG. 1.—Increase in the number of cells and amount of DNA per ml in a partially synchronized culture. To begin the experiment, a logarithmically growing culture was diluted from 7.3×10^5 to 2×10^5 cells per ml with fresh medium at 37°C containing 10^{-6} *M* 5-fluorodeoxyuridine. 0.01 volume of 4.1×10^{-3} *M* thymidine was added at 15 hr (arrow). DNA was assayed fluorometrically on quadruplicate aliquots as previously.[4]

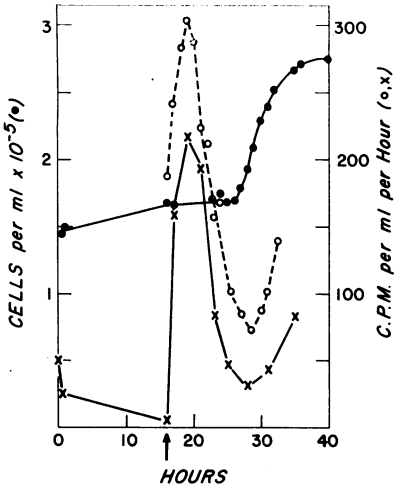

FIG. 2.—Rates of DNA synthesis per ml per hr in a partially synchronized culture. To begin the experiment, a logarithmically growing culture was diluted from 3.8×10^5 to 1.45×10^5 cells per ml with fresh medium at 37°C containing 10^{-6} *M* 5-fluorodeoxyuridine. 0.01 volume of 4.1×10^{-3} *M* thymidine was added at 16 hr (arrow). At the times indicated (×), duplicate 1 ml aliquots were incubated separately for 1 hr with hypoxanthine-8-C^{14} and the radioactivity incorporated into alkali-resistant acid-insoluble material determined (see *Experimental*). Also shown are the rates of DNA synthesis in another culture (○) in which the aliquots (already containing 4.1×10^{-5} *M* thymidine) were incubated with 0.1 volume of 4.1×10^{-4} *M* thymidine-2-C^{14} (6.4 μc per μM) as previously.[4] In this case, the rate at 16 hr represents the first hour after release, rather than before, as with hypoxanthine-8-C^{14}.

averaged 12.9 μμg. Therefore, the logarithmically growing and nonsynchronized cells to which 5-fluorodeoxyuridine was added contained *on the average* 1.2 times the basal amount.[12] Those cells which divided before thymidine release presumably had completed most or all of their DNA synthesis before 5-fluorodeoxyuridine was added.

In Figure 2 are shown for such a culture the rates of DNA synthesis per hr, determined by incubation of aliquots with hypoxanthine-8-C^{14} and subsequent analysis of radioactivity incorporated into alkali-resistant, acid-insoluble material (see *Experimental*). Also shown in Figure 2 are the rates of incorporation into DNA of thymidine-2-C^{14} in a similar culture; in this case, the rate at 16 hr represents the rate for the first hour after release, rather than before, as with hypoxanthine-8-C^{14}. Similar findings have been reported recently by Paul and Hagiwara.[13]

An increasing rate of DNA synthesis over the three hr subsequent to thymidine release, such as that shown in Figure 2, has been studied in HeLa cells by Mueller and co-workers, who have demonstrated that almost all the cells show this increasing rate of synthesis, that it correlates with synthesis at specific chromosomal sites, and that it can be prevented by puromycin.[14, 15]

Changes in DNA polymerase activity: The DNA polymerase activity of the supernatant and particulate fractions of the cell (6,000 × *g*

TABLE 1
CHANGES IN POLYMERASE ACTIVITY FROM BEFORE TO THREE HOURS AFTER THYMIDINE RELEASE

	Supernatant Fraction — cpm in DNA/μg of protein			Particulate Fraction — cpm in DNA/μg of protein		
Experiment	Before release	After release	Per cent change	Before release	After release	Per cent change
1	1.86	1.19	−36	—	—	—
2	2.34	1.23	−47	—	—	—
3	1.53	1.46	−5	—	—	—
4	1.54	1.18	−23	—	—	—
5	2.12	1.64	−23	0.27	0.32	+19
6	1.80	1.76	−2	0.21	0.25	+19
7	2.04	1.42	−30	0.31	0.44	+42
8	1.46	1.61	+10	0.43	0.44	+2
9	2.31	1.22	−47	0.34	0.34	0
10	2.23	1.37	−39	0.35	0.50	+43
11	1.22	1.37	+12	0.33	0.40	+21
Mean	1.86	1.40	−21	0.32	0.38	+21
S.E.mean			±6.5			±6.4

for 30 min) was examined in cells collected before, during, and after the period of DNA synthesis. In 11 consecutive similar experiments, the supernatant fraction showed an average drop in polymerase activity of 21 per cent from just before thymidine release to three hr afterwards (Table 1). This drop is significant (Student's t test yields $0.001 < P_{n=10} < 0.01$). During the same interval, the average content of protein per cell rose 14 per cent; therefore, a decreased synthesis of polymerase might be responsible for the drop in specific activity. However, three experiments in which consecutive samples were assayed indicated a prompt return to the original specific activity after completion of DNA synthesis (Fig. 3).

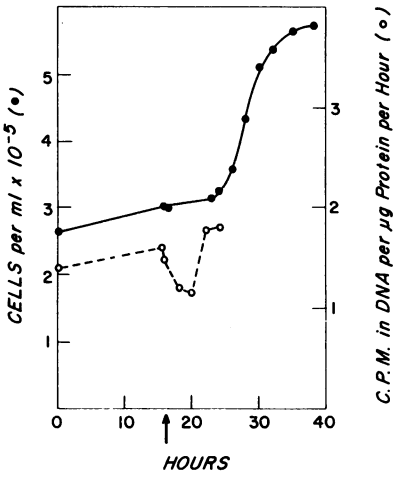

FIG. 3.—Change in DNA polymerase activity in the supernatant fraction of partially synchronized cells. To begin the experiment, a logarithmically growing culture was diluted from 4.5×10^5 to 2.6×10^5 cells per ml with fresh medium at 37°C containing 10^{-6} M 5-fluorodeoxyuridine. 0.01 volume of 4.1×10^{-3} M thymidine was added at 16 hr (arrow). At the times indicated, duplicate aliquots of cells were removed and kept at 4°C for about 24 hr. Then the cells were disrupted and centrifuged, and polymerase activity in the supernatant fractions was determined and averaged (see *Experimental*).

As shown for other cells by previous workers,[16, 17] the particulate fraction was less active than the supernatant fraction (Table 1). Before release, the particulate fraction contained an average of 11 per cent of the cell polymerase activity, as well as 42 per cent of the protein and about 60 per cent of the DNA. Nonetheless, in seven experiments an average increase of 21 per cent in the polymerase activity of the particulate fraction occurred from just before to three hr after release (Table 1). This rise is almost as significant as the decrease in supernatant activity above (Student's t test yields $0.01 < P_{n=6} < 0.02$). In these experiments, the average absolute increase in particulate activity was 9 per cent of the decrease in supernatant activity.

It seemed possible that the rise in particulate polymerase activity was due to residual intact cells or nuclei, which would have increased in activity as shown in Figure 2. Microscopic examination indicated that after disruption an average of 0.05 per cent of the cells remained intact. The nuclei of about 0.2 per cent more of the original cells were recognizable, although distorted. If whole cells and nuclei incorporated d-GTP-8-C^{14} as well as hypoxanthine-8-C^{14}, residual contamination of this magnitude could have accounted for about 5 per cent of the average particulate activity observed. In fact, when tested in the polymerase assay, whole cells incorporated d-GTP-8-C^{14} at about 25 per cent of the rate of incorporation of hypoxanthine. Furthermore, added DNA was not required for incorporation by whole cells, as it was for incorporation by the supernatant or particulate fractions.

Discussion.—In contrast to the intranuclear location of DNA, the studies of Bollum and Potter[16] and Smellie and co-workers[17, 18] have shown that most of the DNA polymerase activity of various mammalian cells is in the soluble fraction of the cell. The present experiments, with those of Gold and Helleiner,[5] indicate

that during DNA synthesis in L cells which are partially synchronized a decrease occurs in the polymerase activity of the supernatant fraction, while an increase usually occurs in particulate activity. These changes might be unrelated; the latter might represent the facilitation, for some reason, of polymerase enzyme already present prior to DNA synthesis in organized subnuclear particles. A more plausible interpretation is that supernatant polymerase enzyme becomes particulate at the time of DNA synthesis and that very likely it is the latter form which is active in the cell. Admittedly, the increase in particulate activity accounted for only 9 per cent of the decrease in supernatant activity. However, studies with certain other systems indicate that an enzyme when particulate may appear less active than when it is soluble.[19-22] Indeed, if the above reasoning is correct, the fact that the average per cent decrease in supernatant activity and increase in particulate activity were the same suggests that the amount of polymerase enzyme protein in these fractions may be similar. If so, the evidence that 11 per cent of the cell polymerase activity was particulate and that the increase in particulate activity accounted for only 9 per cent of the decrease in supernatant activity suggests that, in terms of the usual assay conditions, the enzyme was about 10 per cent as active when particulate as when it was in the supernatant fraction.

Summary.—When suspension cultures of mouse fibroblasts were partially synchronized with 5-fluorodeoxyuridine, DNA synthesis was most active three hr after release of the 5-fluorodeoxyuridine block with thymidine. DNA polymerase activity in the supernatant fraction of the cell decreased 21 per cent from just before thymidine release to three hr after release. During the same period, polymerase activity in the particulate fraction increased 21 per cent, although the absolute amount of increase accounted for only 9 per cent of the decrease in supernatant activity. These results suggest that at the time of DNA synthesis supernatant polymerase became particulate and less active in terms of the usual assay conditions.

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THYRO-ACTIVITY IN IODO-DERIVATIVES OF PHYTOHORMONAL 5-HYDROXYBENZOFURANS

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The thyronines, especially thyroxine and 3,3′,5-triiodothyronine, have been shown to act as antioxidants in test oxidation systems *in vitro*.[1] Among the factors of quantitative significance in determining the level of antioxidant activity, the presence of a phenyl ether skeleton (or phenoxy group) was foremost and the extent of iodination second.[1, 2]

The previously unnoticed antioxidant activity of thyroxine is consistent with its ability to protect the succinoxidase system against oxygen poisoning[3] and, even more directly, with its ability to maintain a high sulfhydryl/disulfide ratio in the mammalian liver.[4]

Antioxidant activity had also been recognized in the indole hormones and a common basis for this property postulated in both plant and animal substances.[5]

Efforts were subsequently undertaken to devise a single structure which would embody the requirements for phytohormonal and thyro-hormonal activities. The structure conceived was that of a benzofuran, specifically derivatives of 2-methyl-3-carbethoxy-5-hydroxybenzofuran (Fig. 1). This structure may be regarded as (*a*) an indole analog, (*b*) a phenoxy acid, (*c*) a hydroxyphenyl ether. If X_1 and/or X_2 are iodine, the thyroxine-like character is obvious.

When the benzofurans were tested against seeds of turnip, alfalfa, and rye, they were indeed found to be highly active as stimulants of germination and seedling growth at concentrations of 10^{-6} to 10^{-4} *M*.[6] The mono-iodinated benzofurans retained phytohormonal properties, but the 4,6-diiodo derivative was completely inactive. Other studies which will be detailed separately have disclosed stimulatory action in a range of processes from leaf expansion to rooting in cuttings.

We have now verified the hypothesis that the iodination of either or both positions *ortho* to the phenolic group confers thyro-activity by demonstrating the hastening of amphibian metamorphosis by these compounds.

Experimentation and Results.—One-year-old tadpoles of the bullfrog, *Rana catesbiana*, were used in the assay for thyro-activity.[7] They were maintained in shallow aerated tapwater (pH ca. 6) in polyethylene pans at 22–23°C in ordinary laboratory light and were fed once weekly a mixture of spinach, lettuce, and boiled eggyolk.

Duplicate vessels of about 20 animals each (in 5 liters of water) were used for