Synthesis of Deoxyribonucleic Acid by Isolated Liver Nuclei*

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SUMMARY

The incorporation of \(^3\)H-TTP into the DNA of intact nuclei isolated from the livers of normal and partially hepatectomized rats has been studied. The regenerating nuclei incorporate \(^3\)H-TTP at about 10 times the rate of normal nuclei. The requirements for a maximal rate of incorporation include the three unlabelled deoxynucleoside triphosphates, ATP, and Mg\(^{++}\). Three pieces of evidence are presented to show that the replication of DNA by the isolated regenerating nuclei is a continuation of the process that was going on in vivo. First, with one exception, a constant relationship exists between the abilities of the nuclei to form DNA in vitro and in vivo after various manipulations of the partially hepatectomized rat. The exception is in the responses of the nuclei after treatment of the rat with cycloheximide. Second, only nuclei that were forming DNA in vivo are able to incorporate \(^3\)H-TTP in vitro. Finally, the isolated nuclei use few or no improper sites of initiation of synthesis. Most or all of the DNA formed is added to polydeoxynucleotide strands that were growing in vivo. The possibility is considered that one of the differences between normal and regenerating nuclei is that the nonreplicating nuclei lack sites of initiation for DNA synthesis. Treatment of normal nuclei with pancreatic DNase causes a marked stimulation in \(^3\)H-TTP incorporation.

At the level of our knowledge, it is not now possible to set criteria for biologically meaningful DNA as it is made by a cell-free animal system. Nonetheless, one property that can be assigned with some certainty relates to the points of initiation of synthesis. For the process in vitro to yield a biologically meaningful product, synthesis must not begin at random points on the genome. Initiation should occur either at the same points as are used by the intact cell or the newly formed DNA should represent only an elongation of the polydeoxynucleotide strands that were being formed in the cell.

It is most unlikely that only proper points of initiation are used in the synthesis of DNA by any soluble preparations of animal enzymes and DNA templates that are now available. Several reports have appeared on the formation of DNA by isolated animal cell nuclei (16-20). In only one of these studies (16, 20), however, have attempts been made to provide evidence for the meaningfulness of the product.

The purpose of this report is to describe a nuclear system from the liver of the partially hepatectomized rat that synthesizes DNA and to present the evidence for the biological meaningfulness of the product.

EXPERIMENTAL PROCEDURE

Materials—Labeled compounds were from New England Nuclear; unlabeled substrates and pancreatic DNase (electrophoretically purified) were from Sigma. Actinomycin D (Dactinomycin) was a product of Merck Sharp and Dohme and cycloheximide (Acti-dione) was from Upjohn.

Treatment of Rats—Male albino rats, obtained locally, were freely given food and water and were used when they weighed about 100 g. Partial hepatectomy refers to the removal of about 70% of the liver (left lateral and median lobes) (21). Unless otherwise indicated, all injections were made in the tail vein and liver samples were taken at 19 to 21 hours after partial hepatectomy.

Isolation of Liver Nuclei—All steps were carried out as rapidly as possible, at 0°. Liver samples (about 1 g) were rinsed in 0.3 M sucrose and they were suspended in 15 to 20 volumes of 0.3 M sucrose-4 × 10⁻³ M CaCl₂ with one stroke in a loose Dounce homogenizer. The suspension was then homogenized (80 strokes) with a loose fitting rubber pestle (22), the homogenate was passed through a monofilament nylon screen (110 mesh), and does not involve improper points of initiation. The term has already been applied in this sense to distinguish between RNA synthesis in vitro that involves proper and improper sites of initiation (15).
the filtrate was centrifuged at 750 × g for 4 min. The supernatant fluid was discarded and the pellet was resuspended in 18 ml of 2 M sucrose-10−3 M CaCl₂ with five strokes in a loose Dounce homogenizer. The suspension was then layered over 13 ml of 2.5 M sucrose and they were sown in 2.5 ml of 0.3 M acetic acid (10%). The DNA was then thrice dissolved (0.3 ml and the final precipitate, dissolved in 1 ml of 1 M NaOH, was heated at 80°C for 15 min. Finally, the DNA was precipitated with trichloroacetic acid on a pad of Celite and the precipitate was washed with acid, ethanol, and ether. DNA was estimated with diphenylamine (23) and radioactivity was measured in a Hydroxy-toluene liquid scintillation mixture.

To measure DNA synthesis in vivo, DNA was purified from isolated nuclei by the method of Zamenhof (24). The purified DNA was precipitated with trichloroacetic acid on a pad of Celite and it was washed and counted as for the estimation of DNA synthesis in vitro.

In double isotope counting, the overlap of ³H into the ¹⁴C channel was negligible; the overlap of ¹⁴C into the ³H channel was 8%.

**RESULTS**

**General Properties of Nuclear Systems**

**Kinetics of ³H-TTP Incorporation by Normal and Regenerating Nuclei**—The kinetics of DNA synthesis by nuclei isolated from normal and regenerating rat liver are shown in Fig. 1. As can be seen from the figure, in the complete mixtures containing all four deoxynucleoside triphosphates, the initial rate of synthesis by the regenerating nuclei was about 10 times that of the normal nuclei. In the incomplete mixtures that lacked the three unlabeled deoxynucleotides, little incorporation of ³H-TTP occurred and no difference was found between normal and regenerating nuclei.

The figure also shows that the normal and regenerating nuclei incorporated ³H-TTP into DNA at nearly linear rates for the first 5 min of incubation, whereupon the rates fell off markedly. The cause of the fall was not established. It did not seem to be due to the removal of a substrate since the same kinetic patterns of incorporation were observed with test mixtures that contained about 30 and 250 µg of DNA as intact normal or regenerating nuclei (also see Table II).

Fig. 1 does not show the results that were obtained when ³H-dATP was used as the labeled substrate. The levels and kinetics...
Nuclear DNA synthesis in vivo and in vitro as function of time after partial hepatectomy

Partially hepatectomized rats were given 1 μCi of [3H]-thymidine at the times indicated. After 30 min, liver samples were removed and nuclei were prepared. The isolated nuclei were incubated with [3H]-TTP in complete mixtures for 5 min as described under "Experimental Procedure." The values for the synthesis of DNA in vitro have been corrected for the incorporation in incomplete mixtures from which the three unlabeled deoxynucleoside triphosphates had been omitted. The data shown are the averages of the results obtained, and the ranges and the numbers of individual determinations are given in parentheses.

<table>
<thead>
<tr>
<th>Time after partial hepatectomy (hrs)</th>
<th>DNA synthesis</th>
<th>In vivo</th>
<th>In vitro</th>
<th>In vitro/in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/mg DNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>450 (230-920)</td>
<td>1,380 (1,060-2,070)</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>350 (190-690)</td>
<td>1,110 (700-1,910)</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>16,100 (9,400-19,700)</td>
<td>26,900 (20,500-34,600)</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>4,220 (3,880-5,100)</td>
<td>5,940 (3,110-6,870)</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

Table IV

Effects of treating partially hepatectomized rats with actinomycin D or x-rays on liver DNA synthesis in vivo and in vitro

All animals received 20 mg of chloramphenicol subcutaneously at zero time. Actinomycin D (5 μg per injection) was given at 0, 1, and 9 hours after partial hepatectomy and whole body x-irradiation (1600 roentgens) was at 1 hour after the operation. At 20 hours after partial hepatectomy, each rat received [3H]-thymidine (9.76 μCi), and liver samples were taken 30 min later. A portion of the isolated nuclei was incubated with [3H]-TTP in the complete mixture for 5 min as described under "Experimental Procedure," and the remaining portion was used to estimate the DNA formed in vivo. The values for the synthesis of DNA in vitro were corrected for the incorporation in incomplete mixtures that lacked the three unlabeled deoxynucleotides. The data shown are the averages of the results obtained with three animals and the ranges of the individual determinations are given in parentheses.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DNA synthesis</th>
<th>In vivo</th>
<th>In vitro</th>
<th>In vitro/in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/mg DNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>11,100 (8,470-12,100)</td>
<td>23,100 (16,800-36,700)</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>230 (70-410)</td>
<td>1,050 (270-1,830)</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>X-irradiation</td>
<td>1,280 (930-1,640)</td>
<td>2,830 (2,410-3,300)</td>
<td>2.2</td>
<td></td>
</tr>
</tbody>
</table>

of incorporation by the isolated nuclei were the same as with [3H]-TTP.

Relationships between Rates of DNA Synthesis and Amounts of Normal and Regenerating Nuclei—Under the conditions of the assay, the incorporation of [3H]-TTP in 4 min was proportional to the levels of normal and regenerating nuclei over at least a range of 20 to 240 µg of nuclear DNA (Table I).

Deoxynucleoside Triphosphate and Other Requirements—For maximal incorporation of [3H]-TTP, normal and regenerating nuclei required the three unlabeled deoxynucleoside triphosphates, ATP, and Mg2+ (Table I). The table shows that the reaction mixtures did not contain limiting concentrations of the unlabeled deoxynucleotides or ATP since reductions in the levels of these constituents had little effect on the incorporation of [3H]-TTP.

The table does not show the incorporation of [3H]-TTP in mixtures in which dCTP was replaced with dCDP or dCMP, one of the deoxynucleotides was replaced with the corresponding ribonucleoside triphosphate, and ADP or AMP was used in place of ATP. Tested with regenerating nuclei, dCDP completely substituted for dCTP but dCMP had no detectable activity; CTP and GTP (1 mm) did not inhibit the incorporation of [3H]-TTP, nor could they replace the corresponding deoxynucleoside triphosphate; and ADP and AMP were not able to substitute for ATP.

Finally, the effects of Spinco supernatant preparations of liver were also studied. The soluble fraction from both normal and regenerating liver enhanced the incorporation of [3H]-TTP by normal and regenerating nuclei by about 50%.

Comparison of Nuclear DNA Synthesis in Vivo and in Vitro

DNA Synthesis in Vivo and in Vitro as Function of Time after Partial Hepatectomy—With the rats used in these studies, the rise in liver DNA synthesis in vitro after partial hepatectomy began at about 12 hours after the operation and reached a peak about 8 hours later (25). The table shows the averages of the results obtained with three animals and the ranges of the individual determinations are given in parentheses.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DNA synthesis</th>
<th>In vivo</th>
<th>In vitro</th>
<th>In vitro/in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/mg DNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>11,100 (8,470-12,100)</td>
<td>23,100 (16,800-36,700)</td>
<td>2.1</td>
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<tr>
<td>Actinomycin D</td>
<td>230 (70-410)</td>
<td>1,050 (270-1,830)</td>
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</tr>
<tr>
<td>X-irradiation</td>
<td>1,280 (930-1,640)</td>
<td>2,830 (2,410-3,300)</td>
<td>2.2</td>
<td></td>
</tr>
</tbody>
</table>
Rats were given cycloheximide (0.5 mg) at 20 hours after partial hepatectomy and were then divided into two groups. Liver samples were removed from the animals of the first group after 10 or 30 min, as indicated. At 10 or 30 min after the antibiotic, the animals of the second group were pulsed with $^3$H-thymidine (10 $\mu$Ci) for 5 min. The nuclei from the unlabeled livers were incubated for 5 min in the complete mixtures containing $^3$H-TTP as described under "Experimental Procedure." The data for DNA synthesis in vitro have been corrected for the incorporation with incomplete mixtures that lacked the three unlabeled deoxy-nucleotides. The labeled liver samples were used to estimate DNA synthesis in vitro. The values shown are the averages of the results obtained, and the ranges and the number of individual determinations are given in parentheses.

Table V  

<table>
<thead>
<tr>
<th>Length of cycloheximide treatment</th>
<th>DNA synthesis</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>cpm/mg DNA</td>
<td>In vivo</td>
<td>In vitro</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>4,080 (2,200–5,970) (5)</td>
<td>28,900 (21,700–35,700) (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>250 (50–580) (5)</td>
<td>20,700 (14,700–27,300) (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>70 (30–140) (4)</td>
<td>10,500 (7,780–18,400) (4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table VI  

Radioautographic analysis of nuclei labeled in vivo and in vitro  

A rat was given 40 $\mu$Ci of $^3$H-thymidine at 20 hours after partial hepatectomy and the liver remnant was removed after 25 min. The isolated nuclei were divided into three portions: the first portion was used to estimate the DNA formed in vivo; the second portion was fixed in acetic alcohol for radioautography; and the last portion was incubated for 5 min in the complete mixture with $^3$H-TTP as described under "Experimental Procedure" (the specific activity of the $^3$H-TTP was increased to 1.5 Ci per mmole). After incubation with $^3$H-TTP, some of the nuclei were fixed in acetic alcohol and the remainder were used to determine the total radioactivity in DNA. The fixed nuclei were spread on microscope slides and coated with Kodak Nuclear Track Emulsion, type NTB 2. To estimate the percentage of labeled nuclei, 1150 ("in vivo") and 1370 ("in vivo and in vitro") nuclei were scored. Each value for the "grains per nucleus" is the average of the results with 50 nuclei (background was taken to be 1 grain per nucleus) and the ranges of the grain counts with individual nuclei are given in parentheses.

<table>
<thead>
<tr>
<th>Stage of labeling</th>
<th>DNA formed</th>
<th>Labeled nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/mg DNA</td>
<td>% total</td>
</tr>
<tr>
<td>In vivo</td>
<td>78,000</td>
<td>14.8</td>
</tr>
<tr>
<td>In vivo and in vitro</td>
<td>152,000</td>
<td>15.1</td>
</tr>
</tbody>
</table>

found to stop about 5 min after the rat was given an inhibitor of protein synthesis (29). Unexpectedly, as Table V shows, preliminary treatment of the partially hepatectomized animal with cycloheximide for 10 min had little effect on the ability of the isolated liver nuclei to synthesize DNA. When the animals were treated with the antibiotic for 30 min, however, the liver nuclei were only about one-third as active as the control nuclei. The nature of the defect in nuclei from animals that had been exposed to cycloheximide for 30 min was not determined.

Evidence for Biologically Meaningful DNA  

Radioautographic Analysis of Nuclei Labeled in Vivo and in Vitro—Partial hepatectomy does not cause a highly synchronized response in DNA replication by the residual liver cells. With the rats used in these studies, 10 to 20% of the liver nuclei synthesized DNA in vivo at 20 hours after partial hepatectomy. It was important to learn whether the same nuclei were responsible for the incorporation of $^3$H-TTP in vitro.

To test this point, a partially hepatectomized animal was given $^3$H-thymidine, and, after 30 min, liver nuclei were isolated and allowed to incorporate $^3$H-TTP in the complete test mixture. The proportions of nuclei that incorporated the radioactive substrates and the degrees of nuclear labeling in vivo and in vitro were then measured radioautographically (Table VI). The data shown in the table indicate that only nuclei that were replicating their DNA in the animal were capable of incorporating $^3$H-TTP.
The conditions were as described for Fig. 1 except that pancreatic DNase (2 µg) was added to some of the reaction mixtures, as shown. The mixtures received averages of 102 µg (normal) or 68 µg (regenerating) of DNA as intact nuclei and incubation was for 5 min. The values shown are the averages of the results of five experiments and the ranges of the individual determinations are given in parentheses.

| Nuclei          | DNase | ³H-TTP incorporation |             |           |
|-----------------|-------|----------------------|-------------|
|                 |       | µmole/mg DNA in 5 min | Complete    | Incomplete| ∆          |
| Normal          | −     | 6.5 (4.0-8.7)        | 0.6 (0.5-0.7)| 0.9       |
|                 | +     | 54 (42-68)           | 32 (21-43)  | 22         |
| Regenerating    | −     | 72 (58-87)           | 2.2 (1.1-2.9)| 70         |
|                 | +     | 157 (121-177)        | 64 (49-71)  | 92         |

Thus, incorporation of ³H-TTP in vitro did not alter the percentage of labeled nuclei, whereas the number of grains per nucleus was increased about 2-fold.

Density Gradient Analysis of DNA Formed in Vivo and in Vitro—When a partially hepatectomized rat incorporated ³H-thymidine in the presence of BUdR, the DNA formed by the liver nuclei was denser than the bulk of the hepatic DNA (Fig. 2a). DNA synthesized in vivo in the absence of BUdR had the same density as the bulk DNA (Fig. 2b).

To determine whether the isolated nuclei elongated the DNA that was being made in the animal, a partially hepatectomized animal was given BUdR, the liver remnant was removed after 10 min, and the isolated nuclei were allowed to incorporate ³H-TTP in the complete reaction mixture. The DNA was then purified, sheared extensively, denatured with heat, and centrifuged in a gradient of CsCl (Fig. 2c). As Fig. 2c shows, the ³H-labeled DNA synthesized by the isolated nuclei was denser than the bulk of the DNA and, therefore, must have been closely associated with the BUdR-labeled DNA that had been made in vivo. DNA formed in vitro by nuclei that had not previously incorporated BUdR had the same density as the bulk of the nuclear DNA (Fig. 2d).

The results with BUdR could also be explained if the analogue or a phosphorylated derivative of it remained in the nuclei during the isolation procedure and was then incorporated in vitro along with ³H-TTP. The following observations were not consistent with this possibility. First, ³H-BUdR was incorporated into liver DNA in vivo but it was not taken up by the isolated nuclei either in the presence or absence of unlabeled TTP. Second, little or no free BUdR (or derivatives) was present in the isolated nuclei. Thus, nuclei (100 µg of DNA) that had been labeled in vivo with ³H-BUdR (0.018 µmole; 1.5 mCi per µmole; 10-min pulse period) were contaminated with less than 0.01 µmole of cold perylcholine acid-soluble BUdR (or derivatives) although 1.4 µmole of the analogue were present in the DNA. To become incorporated into nuclear DNA during incubation in vitro, the low level of BUdR would have had to compete with the 3 µmole of TTP present in the test mixture.

Effects of Pancreatic DNase on ³H-TTP Incorporation by Isolated Normal and Regenerating Nuclei—One explanation for the low incorporation of ³H-TTP by the isolated nuclei was that they were deficient in points at which DNA synthesis could be initiated. To examine this possibility, pancreatic DNase was added to the test mixtures (Table VII). As can be seen from the table, the nucleases caused a marked stimulation of ³H-TTP incorporation both in the complete mixture and in the mixture that lacked the three unlabeled deoxynucleotides. For comparison, the table also shows the results that were obtained with regenerating nuclei.

The table does not show that almost all of the ³H-TTP incorporated in the presence of pancreatic DNase was associated with the nuclei. Thus, in a typical experiment, after 5 min of incubation in the complete test mixture containing DNase, 2240 cpm were recovered in the sedimented normal nuclei (600 x g, 4 min) and only 120 cpm were found in the supernatant fluid.

### Discussion

Three pieces of evidence have been presented in support of the biological meaningfulness of the DNA synthesized by nuclei isolated from regenerating rat liver. First of all, with one exception, there is a constant relationship between the nuclear activities in vitro and in vivo after various manipulations of the animals. Second, only those nuclei that were forming DNA in the animal were able to incorporate ³H-TTP in vitro. Finally, the isolated nuclei use few or no improper sites of initiation. Most or all of the DNA made by them is added to polydeoxynucleotide chains that were growing in vivo.

Evidence for the elongation of the DNA that was being formed in the animal was obtained with equilibrium centrifugation. The ³H-TTP incorporated by the isolated nuclei was shown to be in close association with the BUdR-labeled DNA (about 0.1% of the total DNA) that was being synthesized in vivo. It is important to point out that the doubly labeled DNA was extensively sheared and denatured with heat before centrifugation. These steps are critical to the demonstration of the continuous nature of the replicative process in vivo and in vitro. With the omission of either step, a similar banding of the dense and radioactive DNA fractions can only be interpreted to mean that the same nuclei had incorporated both BUdR and ³H-TTP.

The single exception to the similar activities of the liver nuclei in vivo and in vitro is in their abilities to form DNA after treatment of the animal with cycloheximide. Liver DNA formation in vivo has been shown to stop about 5 min after the partially hepatectomized rat is given cycloheximide or other inhibitors of protein synthesis (29). However, nuclei from the livers of the treated animals show little or no loss in activity in vitro.

The mechanism by which inhibitors of protein synthesis block the continuation of liver DNA replication in vivo is not known. One possibility is that some protein is required for DNA synthesis or for the stabilization of nascent DNA. If this is so, the isolated nuclei may be able to form DNA because they can provide the essential protein through leaching from the chromatin.

The results of some nuclear transplantation and cell fusion experiments with animal cells have implicated changes in the cytoplasm as being responsible for stimulating the nuclei to enter the period of DNA replication (9-12). The observations made with the isolated liver nuclei are not in conflict with these results.

The large difference in the activities of normal and regenerating nuclei in vitro could be explained, for example, if nonreplicating nuclei lack initiation points for DNA synthesis and if these points...
are somehow produced through the action of the regenerating liver cytoplasm. Once DNA synthesis is begun in vivo, the process would be able to continue even after the nuclei are separated from the cytoplasm.

Some support for the idea that nonreplicating liver nuclei lack points of initiation for DNA synthesis comes from the experiment with pancreatic DNAse. The addition of the nuclease to test mixtures containing normal nuclei produces a large increase in the incorporation of $^3$H-TTP. Clearly, the normal nucleus has a connection, it can be pointed out that an intact duplex circle or an intact linear duplex cannot serve as a template or primer for purified Escherichia coli DNA polymerase (30). It is also of interest that breaks in the DNA template made by endonucleases provided DNA that has improper points for initiation. In this experiment, it would be able to continue even after the nuclei are separated from the cytoplasm.

The nuclear systems from liver should be excellent tools for looking for the cytoplasmic changes that lead to DNA replication. In this connection, preliminary efforts have been made to enhance synthesis in normal nuclei (and in nuclei taken from partially hepatectomized rats before the rise in DNA synthesis begins) with the soluble fraction from regenerating liver. Some stimulation was found, but it was small, and the soluble fraction from normal liver gave the same enhancement in activity.

The difference in the abilities of normal and regenerating nuclei to make DNA in vitro provides confidence that the nuclei can provide confidence that the nuclei can provide DNA that has improper points for initiation. In vivo. Partial hepatectomy when DNA synthesis in vivo is at its peak.

The presence of latent DNA polymerase activity in the normal nuclei (140 $\mu$g of nuclear DNA) was also shown by adding native and heat-denatured calf thymus DNA (25 $\mu$g) to complete and incomplete test mixtures. In the complete mixtures, 0.85, 4.8, and 3.5 $\mu$moles of $^3$H-TTP were incorporated in 5 min with no calf thymus DNA and with added native and denatured DNA, respectively. In the incomplete mixtures, the comparable values were 0.09, 1.1, and 0.5.

References

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