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 unlabeled 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 vivo and in vitro 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.

The synthesis of nuclear DNA in animal cells occurs only during a part of the division cycle (1-8). The cellular changes that must take place before replication can begin are unknown. Nuclear transplantation and cell fusion experiments have provided evidence for critical events in the cytoplasm (9-12) and in the nucleus (13, 14). Before the exact nature of these events can be explored, it would seem to be essential to develop cell-free systems that synthesize "biologically meaningful" DNA.

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 unlabeled 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 vivo and in vitro 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.

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† By whom these studies will be submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Anatomy and Cell Biology.

The term "biologically meaningful" does not imply that the newly formed DNA has biological activity. Rather, it is used in a narrow sense to describe DNA the formation of which in vitro 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°C. 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 \(\times 10^{-3} M \) CaCl\(_2\) 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).
Deoxyribonucleoside triphosphate and other requirements for $^3$H-TTP incorporation by isolated nuclei

The conditions were as described in Fig. 1 except that one component of the complete test mixture and, where applicable, from the corresponding mixture that lacked the three unlabeled deoxynucleoside triphosphates ("incomplete") was omitted or its concentration was reduced as shown. Each value shown is the average of the results obtained in three to eight experiments.

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results

General Properties of Nuclear Systems

Kinetics of $^3$H-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 normal and regenerating nuclei is shown in Fig. 1. The conditions were as described in Fig. 1 except that the initial rate of synthesis by the normal and regenerating nuclei was about 10 times that of the normal nuclei.

The figure also shows that the normal and regenerating nuclei incorporated $^3$H-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 were observed with test mixtures that contained about 30 and 250 µg of DNA as intact normal or regenerating nuclei (also see Table I).

Fig. 1 does not show the results that were obtained when $^3$H-dATP was used as the labeled substrate. The levels and kinetics

Table II

Deoxyribonucleoside triphosphate and other requirements for $^3$H-TTP incorporation by isolated nuclei

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RESULTS

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**Table III**

**Nuclear DNA synthesis in vivo and in vitro as function of time after partial hepatectomy**

Partially hepatectomized rats were given 1 mCi of $^{3}H$-thymidine at the times indicated. After 30 min, liver samples were removed and nuclei were prepared. The isolated nuclei were incubated with $^{3}H$-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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vitro</td>
</tr>
<tr>
<td>0</td>
<td>450 (230-920) (5)</td>
</tr>
<tr>
<td>12</td>
<td>350 (190-690) (5)</td>
</tr>
<tr>
<td>20</td>
<td>16,100 (9,400-19,700) (8)</td>
</tr>
<tr>
<td>48</td>
<td>4,280 (3,880-5,100) (3)</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 2 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 $^{3}H$-thymidine (0.75 μCi), and liver samples were taken 30 min later. A portion of the isolated nuclei was incubated with $^{3}H$-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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vivo</td>
</tr>
<tr>
<td><strong>None</strong></td>
<td>11,100 (8,470-12,100)</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>230 (70-410)</td>
</tr>
<tr>
<td>X-irradiation</td>
<td>1,280 (930-1,640)</td>
</tr>
</tbody>
</table>

Effects of incorporation by the isolated nuclei were the same as with $^{3}H$-TTP.

**Relationships between Rates of DNA Synthesis and Amounts of Normal and Regenerating Nuclei**—Under the conditions of the assay, the incorporation of $^{3}H$-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 $^{3}H$-TTP, normal and regenerating nuclei required the three unlabeled deoxynucleoside triphosphates, ATP, and Mg$^{2+}$ (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 $^{3}H$-TTP.

The table does not show the incorporation of $^{3}H$-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 μM) did not inhibit the incorporation of $^{3}H$-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 $^{3}H$-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 vivo after partial hepatectomy began at about 12 hours after the operation and reached a peak about 8 hours later (25). Table III compares the abilities of the liver nuclei to form DNA in vivo and in vitro as a function of time after partial hepatectomy. As the table shows, the two activities were parallel.

**Effects of Treating Partially Hepatectomized Rats with Actinomycin D or X-rays on Liver DNA Synthesis in Vivo and in Vitro**—When partially hepatectomized rats were treated with low levels of actinomycin D (26, 27) or X-rays (28) during the prereplicative period, little or no rise in liver DNA synthesis in vivo occurred at 20 hours after the operation. As can be seen from Table IV, DNA synthesis by the liver nuclei isolated from the treated animals was greatly reduced. The reductions in vitro were comparable to those found in vivo.

**Effects of Treating Partially Hepatectomized Rats with Cycloheximide on Liver DNA Synthesis in Vivo and in Vitro**—Ongoing DNA synthesis by regenerating liver nuclei in vivo was...
TABLE V

Effects of treating partially hepatectomized rats with cycloheximide on liver DNA synthesis in vivo and in vitro

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 mixture 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 deoxyribonucleotides. The labeled liver samples were used to estimate DNA synthesis in vivo. The values shown are the averages of the results obtained, and the ranges and the number of individual determinations are given in parentheses.

<table>
<thead>
<tr>
<th>Length of cycloheximide treatment</th>
<th>DNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vivo</td>
</tr>
<tr>
<td>min</td>
<td>cpm/mg DNA</td>
</tr>
<tr>
<td>10</td>
<td>4,080 (2,200-5,970) (3)</td>
</tr>
<tr>
<td>250 (50-580) (5)</td>
<td>20,700 (14,700-27,300) (7)</td>
</tr>
<tr>
<td>30</td>
<td>70 (30-140) (4)</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 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 grains/nucleus</td>
</tr>
<tr>
<td>In vivo</td>
<td>78,000</td>
<td>14.8 49 (21-69)</td>
</tr>
<tr>
<td>In vivo and in vitro</td>
<td>152,000</td>
<td>15.1 90 (47-124)</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.
Thus, incorporation of \(^3\)H-TTP in \textit{vivo} 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 \(^3\)H-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 \textit{in vivo} in the absence of BUdR had the same density as the bulk DNA (Fig. 2b).

To learn 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 \(^3\)H-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 \(^3\)H-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 \textit{in vivo}. DNA formed \textit{in vitro} by nuclei that had not previously incorporated BUdR had the same density as the bulk 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 \textit{in vitro} along with \(^3\)H-TTP. The following observations were not consistent with this possibility. First, \(^3\)H-BUdR was incorporated into liver DNA \textit{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 \mu g of DNA) that had been labeled \textit{in vivo} with \(^3\)H-BUdR (0.018 \mu mole; 1.5 mCi per \mu mole; 10-min pulse period) were contaminated with less than 0.01 \mu mole of cold p-chloroacetyl-soluble BUdR (or derivatives) although 1.4 \mu moles of the analogue were present in the DNA. To become incorporated into nuclear DNA during incubation \textit{in vitro}, the low level of BUdR would have had to compete with the 8 \mu moles of TTP present in the test mixture.

**Effects of Pancreatic DNase on \(^3\)H-TTP Incorporation by Isolated Normal and Regenerating Nuclei**—One explanation for the low incorporation of \(^3\)H-TTP by the isolated normal 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 nuclease caused a marked stimulation of \(^3\)H-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 \(^3\)H-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 \times 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 \textit{in vitro} and \textit{in vivo} after various manipulations of the animals. Second, only those nuclei that were forming DNA in the animal are able to incorporate \(^3\)H-TTP \textit{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 \textit{in vivo}.

Evidence for the elongation of the DNA that was being formed in the animal was obtained with equilibrium centrifugation. The \(^3\)H-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 \textit{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 \textit{in vivo} and \textit{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 \(^3\)H-TTP.

The single exception to the similar activities of the liver nuclei \textit{in vivo} and \textit{in vitro} is in their abilities to form DNA after treatment of the animal with cycloheximide. Liver DNA formation \textit{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 \textit{in vitro}.

The mechanism by which inhibitors of protein synthesis block the continuation of liver DNA replication \textit{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 nucleus 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 \textit{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 latent ability to incorporate $^3$H-TTP that can be expressed by providing DNA that has improper points for initiation. In this 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 E. coli DNA polymerase (30). It is also of interest to note that breaks in the DNA template made by endonucleases stimulate transcription by a bacterial RNA polymerase that has essentially free of $\alpha$ factor (31) and that x-ray damage of DNA also increases the nonspecific initiation of RNA synthesis (15).

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 DNA synthesis by liver nuclei with the soluble fraction from regenerating liver. Some support for the idea that nonreplicating liver nuclei lack points of initiation for DNA synthesis comes from the experiment in which nuclear extracts from normal livers and from livers taken at 20 hours after 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