Stimulation of DNA Polymerase by Factors Isolated from Novikoff Hepatoma*

(in vitro DNA synthesis/protein purification/rat liver/tumor/DNA nucleotidyltransferase)

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Communicated by Henson Swift, January 8, 1975

ABSTRACT Extracts of Novikoff hepatoma cells contain factors capable of stimulating in vitro DNA synthesis several fold. The activity can be resolved into three separate protein peaks on DEAE-Sephadex. Two of these, factors II and III, have been purified and partially characterized. Both factors increase the initial rate of DNA synthesis and allow synthesis to proceed much longer. If either factor is added after synthesis by the DNA polymerase has reached a plateau, resumption of synthesis occurs. The factors appear to have different modes of action or sites of action since they show an additive effect even when a single one is used at saturating conditions. These factors are present in normal rat liver but at a concentration less than 5% of that found in the tumor cells. When tested with several highly purified DNA polymerases (DNA nucleotidyltransferase, EC 2.7.7.7), the factors show a much greater stimulation of homologous, nonmitochondrial enzymes (rat liver nuclear-, rat liver cytoplasmic-, or Novikoff-DNA polymerases) when compared with rat liver or calf liver mitochondrial- Escherichia coli- or sea urchin nuclear-DNA polymerases. The mechanism of action of these factors is not known at present. No enzymatic activity has been associated with factor III. Highly purified, but not homogeneous, preparations of factor II contain low levels of endonuclease; it has not been established whether endonuclease is a contaminant or is responsible for the stimulating activity.

Prokaryotic systems have provided ideal models for the study of DNA replication. In the past few years, it has become clear that replication is a complex process involving the participation of not only DNA polymerase but of several other accessory proteins as well. In Escherichia coli three distinct DNA polymerases have been identified (3–5). A modified form of DNA polymerase III, polymerase III*, appears to be the primary replicating enzyme (6, 7), although polymerases I or II may also be involved in the replication process (8). Polymerase III* requires a specific accessory protein, co-polymerase III* (6), which probably participates in the initiation process (7). From genetic studies, the products of at least six loci are known to be essential for DNA replication (9). The products of the dnaB (10, 11), dnaC-D (12), dnaE (5, 13), dnaF (14), and dnaG (15) loci have all been purified and their role in the replication process is under investigation. Recently two additional proteins have been identified which stimulate in vitro DNA synthesis (16).

In mammalian systems, studies of DNA replication have progressed more slowly, in part, to lack of similar genetic markers. Several lines of evidence, however, have suggested the presence of accessory proteins or factors which may stimulate DNA synthesis. Examples include (i) cell fusion and nuclear transplantation studies where a template-restricted nucleus is stimulated to synthesize DNA when transplanted into an actively dividing cell (17, 18); (ii) the stimulation of DNA synthesis by humoral factors in the blood of animals undergoing liver regeneration (19, 20); and (iii) the presence of stimulatory activity in sera and conditioned media from cultured cells (21).

We have initiated a search for factors in mammalian cells which participate in DNA replication. As a first approach we have looked for proteins which specifically enhance the activity of DNA polymerase. This report identifies three such factors isolated from the Novikoff hepatoma which are active with the homologous, nonmitochondrial DNA polymerases.

MATERIALS AND METHODS

DNA Polymerases. DNA polymerase from isolated rat liver nuclei (22) was purified further on phosphocellulose and hydroxyapatite (23). Cytoplasmic DNA polymerase was purified from the 100,000 × g supernatant of rat liver homogenate. The supernatant (Fraction I) was treated with (NH₄)₂SO₄ and the proteins precipitating at 45–75% saturation (Fraction II) were recovered, dissolved and dialyzed against TMEG–0.01 M NaCl [TMEG buffer: 0.02 M Tris-HCl at pH 8.0, 0.005 M 2-mercaptoethanol, 0.001 M EDTA, 10% (w/v) glycerol]. Fraction II was loaded onto a 4.9 cm × 22 cm column of DEAE-Sephadex A50 equilibrated with TMEG–0.01 M NaCl, and the polymerase activity (Fraction III) eluted as a single peak at 0.12 M NaCl with a 500 ml linear gradient (0.01–0.30 M NaCl in TMEG). Fraction III was dialyzed against PMEG [PMEG buffer: 0.02 M potassium phosphate at pH 7.5, 0.005 M 2-mercaptoethanol, 0.001 M EDTA, 10% (w/v) glycerol], and loaded onto a 1.75 cm × 20 cm column of phosphocellulose equilibrated with PMEG. The enzyme was eluted (Fraction IV) at 0.18 M with a 300 ml linear gradient of 0–0.45 M potassium phosphate in PMEG. Fraction IV was dialyzed against PMG buffer (PMEG with EDTA deleted), and loaded onto a 0.63 cm × 8 cm column of hydroxyapatite equilibrated with PMG. The polymerase activity (Fraction V) was eluted at 0.18 M with a 200 ml linear gradient of 0–0.45 M potassium phosphate in PMG. Fraction V was dialyzed against TMEG buffer, and bovine serum albumin was added to a concentration of 1 mg/ml. This fraction gives two stainable bands on polyacrylamide disc gel electrophoresis. Purification to homogeneity can be achieved with DNA-cellulose chroma-

Abbreviations: TMEG, 0.02 M Tris-HCl at pH 8.0, 0.005 M 2-mercaptoethanol, 0.001 M EDTA, 10% (w/v) glycerol; PMEG, 0.02 M potassium phosphate at pH 7.5, 0.005 M 2-mercaptoethanol, 0.001 M EDTA, 10% (w/v) glycerol; PMG, PMEG with EDTA omitted.

*Preliminary communications of this work have appeared in abstract form (1, 2).
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A*-A* polymerase activity peaks at a flow rate of 45 ml/hr. Each 5 ml fraction was assayed for DNA polymerase under limited substrate conditions as described in Materials and Methods. Each fraction was then re-assayed with the addition of 0.02 µg of Fraction V rat liver cytoplasmic DNA polymerase, which gave approximately 8000 cpm. Stimulatory activity is detected as an increase above this level. The three Novikoff factors eluted at approximately 0.05 M, 0.09 M, and 0.15 M NaCl. Residual DNA polymerase is easily separated from the factors since it elutes between peaks II and III at 0.12 M NaCl. The maximal DNA polymerase activity was only about 0.1 units. —— DNA polymerase; —— stimulatory activity; —— NaCl concentration.

Fig. 1. DEAE-Sephadex elution profile. The 75-85% ammonium sulfate fraction (243 mg) was loaded onto a 4.9 cm x 22 cm column of DEAE-Sephadex A50 equilibrated with TMEG-0.01 M NaCl. After washing with buffer, proteins were eluted with a 500 ml linear gradient of 0.01-0.3 M NaCl in TMEG buffer at a flow rate of 45 ml/hr. Each 5 ml fraction was assayed for DNA polymerase under limited substrate conditions as described in Materials and Methods (approximately 8000 cpm = 1 pmol). Each fraction was then re-assayed with the addition of 0.02 µg of Fraction V rat liver cytoplasmic DNA polymerase, which gave approximately 8000 cpm. Stimulatory activity is detected as an increase above this level. The three Novikoff factors eluted at approximately 0.05 M, 0.09 M, and 0.15 M NaCl. Residual DNA polymerase is easily separated from the factors since it elutes between peaks II and III at 0.12 M NaCl. The maximal DNA polymerase activity was only about 0.1 units. —— DNA polymerase; —— stimulatory activity; —— NaCl concentration.

Fig. 2. Stimulation of rat liver cytoplasmic DNA polymerase by various concentrations of Novikoff factor II and III. To assay tubes containing approximately 11 units of rat liver cytoplasmic DNA polymerase Fraction V (0.035 µg of protein) under standard assay conditions, we added various amounts of Novikoff factor II (stock solution = 1.07 mg/ml) or Novikoff factor III (stock solution = 77 ug/ml) from the pooled and concentrated DEAE-Sephadex peaks. In another set of tubes, saturating levels of factor III (equivalent to 30 µl per assay) were added to the reaction mix with various concentrations of factor II as shown in the figure. ——. A further enhancement of DNA polymerase can be observed.

DNA polymerase from E. coli (24), sea urchin nuclei (25), and rat liver mitochondria (26) were purified by published methods. Calf liver mitochondrial DNA polymerase was purified by modifying the procedure used for the rat liver enzyme (Meyer, unpublished data). Activated calf thymus DNA, rendered 20% acid soluble by DNase I, was prepared as described by Loeb (25).

DNA Polymerase Assays. Unless otherwise noted, all assays were carried out in 125 µl reactions containing 0.025 M Tris-HCl at pH 8.4, 0.005 M 2-mercaptoethanol, 0.0005 M EDTA, 0.010 M MgCl₂, 0.015 mM each of dATP, dCTP, dGTP, and [³H]dTMP (specific activity 325 mCi/mmol), 15% (w/v) glycerol, 100 µg/ml of activated DNA, and 25 µl of DNA polymerase. Incubations were for 1 hr at 37°, and incorporation of [³H]dTMP into trichloroacetic acid-insoluble product on filter paper discs was determined as described previously (27). To detect activity off columns, we used [³H]dTMP undiluted at a concentration of 0.3 mM (specific activity 18 Ci/mmol). For assays of stimulatory activity, 1–20 units of rat liver cytoplasmic or Novikoff polymerase was added to each tube. A unit of DNA polymerase activity is defined as the incorporation of 1 pmol of [³H]dTMP into DNA per hr at 37°. A unit of stimulatory activity is defined as that which causes a 100% increase in the DNA polymerase activity in a standard assay.

Other Methods. Phosphomonoesterase, phosphodiesterase, and nuclease assays were performed as described earlier (26). Protein was determined by the Lowry reaction (28).

Novikoff hepatoma was maintained in Holtzman rats by intraperitoneal injection, and the ascites fluid harvested 6 days postinjection. The fluid was centrifuged for 10 min at 12,000 x g and the cell pellet resuspended in 0.01 M Tris·HCl at pH 7.0, 0.002 M MgCl₂. After hemolysis of the erythrocytes, the tumor cells were collected by centrifugation, washed in Tris-Mg and either frozen at −20° or used immediately for preparation of DNA polymerase and factors.

RESULTS

Identification of Novikoff stimulatory proteins

Washed Novikoff cells were extracted for 2 hr at 0° by the addition of two volumes of 0.02 M Tris·HCl at pH 7.0, 0.005 M 2-mercaptoethanol, 0.0001 M EDTA, 0.2% Triton X-100, and 3.0 M NaCl. The extract was centrifuged for 2 hr at 70,000 x g and dialyzed exhaustively against TMEG-0.15 M NaCl (Fraction I). Fraction I was treated with (NH₄)₂SO₄ and proteins precipitating at 45-75% and 75-85% saturation were recovered, dissolved, and dialyzed against TMEG-0.01 M NaCl. Most of the DNA polymerase activity was recovered in the 45-75% (NH₄)₂SO₄ fraction and was purified to homogeneity (data to be published). The 75-85% (NH₄)₂SO₄ fraction, while having only 2% of the total DNA polymerase activity, contained factors which stimulated purified rat liver nuclear- or cytoplasmic- or Novikoff- polymerase several-fold. When chromatographed over DEAE-Sephadex (Fig. 1), three peaks of activity could be resolved and separated from residual DNA polymerase which elutes between peaks II and III. (For convenience the factors have been identified by their order of elution from DEAE-Sephadex.) Factor I has the least activity and is present in variable amounts; in some preparations it is not detectable at all and has not been investigated further as yet. Peak II has been purified to approximately 50% homogeneity on phosho-
cellulose and hydroxyapatite. At this stage of purification it yields four protein bands in polyacrylamide disc gels, and activity has been localized to one of them. Factor III has been purified to homogeneity on DNA-cellulose. In polyacrylamide gels, there is a single band, and activity has been localized to this protein. Details of the purification and properties of factors II and III will be presented in a later communication. Both factors are nondenatured, are inactivated by heating for 5 min at 100°C and are insensitive to DNase or RNase digestion. They give a negative orcinol or diphenylamine reaction, a positive Folin reaction, and have a maximal UV absorption at 280 nm, indicating they are proteins. In polyacrylamide disc gels, they migrate towards the anode which is characteristic of acidic proteins. At this stage of purification both factors lack detectable RNA polymerase, DNA polymerase, RNA-unwinding, acid or alkaline phosphatase, acid or alkaline phosphodiesterase I, phosphodiesterase II, or ATP-dependent or -independent nuclease activity measured by solubilization of [3H]DNA. There appears to be a very low level of endonuclease associated with factor II, but this activity has not yet been identified as the stimulatory activity (see Discussion). To see whether these factors are unique to tumor tissue, we examined normal liver extracts for such factors. These extracts contained the factors, but at barely detectable levels, and they amounted to less than 5% of that in Novikoff cells.

**Stimulation of DNA polymerase by Novikoff factors**

Addition of Novikoff factors II or III to rat liver cytoplasmic DNA polymerase results in a several-fold increase in DNA synthesis (Table 1, Fig. 2). Table 1 also shows that neither factor is providing primer/template for DNA synthesis nor does either contain endogenous DNA polymerase activity. When both factors are added together, there is a further increase in activity. In order to determine whether the two factors affect the same site or have similar modes of action, we determined the additive effect under saturating conditions. In Fig. 2, factor III shows saturation at approximately 3 units while factor II has not leveled off. From other experiments (not shown) we have found saturation of factor II occurs about 17-20 units under these conditions. When factor III is added at saturating levels and factor II varied (Fig. 2, upper curve), a further increase in activity is seen with shape of the curve roughly parallel to that of factor II. From this we conclude that the mode of action or the site of action of the two factors is probably different.

It should be noted that the stimulation of the DNA polymerase by these factors is not affected by changes in deoxyribonucleoside triphosphate concentration, pH, divalent cations, or Mg2+ concentration, but is reduced at high ionic strengths. Stimulation shows wide variation depending on primer/template. With denatured DNA, a very poor primer/template for these enzymes, stimulation up to 50-fold can be obtained.

![Figure 3](image) Time course of incorporation of [3H]dTMP into DNA by rat liver cytoplasmic DNA polymerase with and without Novikoff factors. Standard assay conditions were as described in Materials and Methods. Each tube contained approximately 45 units of Fraction V rat liver cytoplasmic DNA polymerase (0.15 μg of protein). Without Novikoff factors, synthesis levels off at about 1 hr. Addition of 20 μl of DEAE-Sephadex, peak II (approximately 22 μg of protein) O—O, or 20 μl of peak III (approximately 1.3 μg of protein) Δ—Δ, or 12.5 μl of each O—O, leads to a rapid initial rate of synthesis and then a slower rate which continues beyond the 6 hr shown. Addition of either factor II ■—■ or factor III ▲—▲ to DNA polymerase 2 hr after the start of the experiment (arrow) when incorporation has reached a plateau, leads to a reinitiation of DNA synthesis which does not level off in the 4 hr tested.

**Table 1. Novikoff factors stimulate rat liver cytoplasmic DNA polymerase**

<table>
<thead>
<tr>
<th>Additions or deletions</th>
<th>[3H]dTMP incorporated (pmol)</th>
<th>Percent control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>9.5</td>
<td>100</td>
</tr>
<tr>
<td>+ factor II</td>
<td>30.1</td>
<td>317</td>
</tr>
<tr>
<td>+ factor III</td>
<td>33.6</td>
<td>354</td>
</tr>
<tr>
<td>+ factor II + factor III</td>
<td>46.2</td>
<td>466</td>
</tr>
<tr>
<td>− DNA + factor II</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>− DNA + factor III</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>− DNA + factor II</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>− DNA + factor II</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>− DNA + factor II</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>− DNA + factor II</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>− DNA + factor II</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>− DNA + factor II</td>
<td>0.1</td>
<td>1</td>
</tr>
</tbody>
</table>

Assay conditions were as described in Materials and Methods. For factor additions, 25 μl of DEAE-Sephadex peaks II and III were used. This represents 19 μg and 2.7 μg of protein, respectively. Fraction V cytoplasmic DNA polymerase (0.09 μg per assay) was used as the enzyme source.

**Table 2. Effect of Novikoff factors on several DNA polymerases**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>[3H]dTMP incorporated (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Rat liver</td>
<td></td>
</tr>
<tr>
<td>cytoplasmic</td>
<td>8.1</td>
</tr>
<tr>
<td>Rat liver nuclear</td>
<td>5.1</td>
</tr>
<tr>
<td>Novikoff</td>
<td>21.2</td>
</tr>
<tr>
<td>Rat liver mitochondrial</td>
<td>2.7</td>
</tr>
<tr>
<td>Calf liver mitochondrial</td>
<td>8.3</td>
</tr>
<tr>
<td>Sea urchin nuclear</td>
<td>78.4</td>
</tr>
<tr>
<td>E. coli</td>
<td>20.2</td>
</tr>
</tbody>
</table>

Assay conditions were described in Materials and Methods. For factor additions, 25 μl of Sephadex peaks II or III were used.

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*Proc. Nat. Acad. Sci. USA 72 (1975)*

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obtained, but the total incorporation of nucleotides is far less than with native or activated DNA.

Effect of Novikoff factors on reaction kinetics
With rat liver cytoplasmic DNA polymerase, incorporation levels off by 1 hr (Fig. 3). Addition of either or both factors results in continuation of incorporation for several hours. The kinetics are biphasic and show an initial rapid rate and then a slower linear rate of synthesis. Again, an additive effect is illustrated. Thus, in addition to enhancing the initial rate of synthesis, these factors allow prolonged continuation of synthesis. Interesting also is the effect of adding either factor to the reaction mixture after the polymerase activity has reached a plateau. Both factors are capable of causing a resumption of DNA synthesis. Whether this represents new initiations or continuation of old chains remains to be elucidated.

Effect of Novikoff factors on other DNA polymerases
The effect of the two Novikoff factors was tested with several highly purified DNA polymerases (Table 2). The factors are capable of stimulating other polymerases but only to a limited extent. The data in Table 2 were obtained with the DEAE-Sephadex fractions. When some enzymes were tested with more concentrated factor preparations, stimulation up to 50% was obtained with heterologous DNA polymerases. The homologous enzymes from rat liver nuclei and cytoplasm and from Novikoff hepatoma were all stimulated several-fold. The mitochondrial DNA polymerase from rat liver, however, behaved much like heterologous enzymes in that it showed only a slight stimulation. While the assay conditions were optimal for characterizing the rat cytoplasmic enzyme system, these conditions are not far from optimal for all other enzymes tested except the mitochondrial polymerases. When the factors were tested with mitochondrial enzymes under optimal conditions (26), results comparable to those shown in Table 2 were obtained.

DISCUSSION
Extracts of Novikoff hepatoma contain factors which have been shown to enhance the in vitro activity of DNA polymerase several-fold. The effects of the factors are 3-fold: (i) increase in initial rate, (ii) prolongation of synthesis, and (iii) ability to reinitiate synthesis once the enzyme activity has reached a plateau. Whether these factors have a biological role in DNA replication remains to be determined.

However, the fact that they enhance activity of the homologous enzymes to a much greater extent than heterologous or mitochondrial enzymes, coupled with the fact that they are present in a 20-fold greater concentration in rapidly dividing tumor cells as compared with normal liver, would support such an interpretation.

The mechanism of action at present is unknown. Factor III has no demonstrable enzyme activity but can bind to DNA polymerase (23). Factor II in our most purified fraction contains low levels of endonuclease activity. Whether specific endonuclease is a contaminant or the actual stimulatory activity can be determined after purification to homogeneity. It does not seem likely that endonuclease action is responsible for stimulation since the primer/template has been maximally activated already by the endonuclease DNase I. Further treatment of this DNA with DNase I leads to less template/priming ability. However, should factor II prove to be endonuclease, it must be attacking limited but specific sites favorable to the DNA polymerase.

We wish to thank Dr. Harris Busch, Baylor University Medical Center, Houston, for supplying original stocks of the Novikoff hepatoma. We are also grateful to Dr. Lawrence Loeb, Institute for Cancer Research, Philadelphia for the gift of homogeneous E. coli and sea urchin DNA polymerases. We thank Drs. Stephen Keller and John Trela for many helpful discussions. This work was initiated under Grant NP-24 from the American Cancer Society.