Effect of Phenylhydrazine-induced Hemolytic Anemia on Nuclear RNA Polymerase Activity of the Mouse Spleen

By Jerry L. Spivak, Dennis Toretti, and Herbert W. Dickerman

The DNA-dependent RNA polymerase of nuclei from lymphoid-rich and erythroid-rich mouse spleens was compared in regard to requirements and conditions of the reactions. The inhibition by either actinomycin D or pancreatic DNase indicated that a DNA template was required for the observed reactions. Following the induction of a hemolytic anemia by the administration of phenylhydrazine, there was a tenfold increase in the nuclear polymerase activity per milligram nuclear DNA of the developing erythropoietic spleen when the assays were done at low ionic strength and more than a threefold increase at high ionic strength. The peak rise in polymerase activity precedes the maximal development of the erythropoietic spleen by 3 days.

The response of the mouse spleen to a phenylhydrazine-induced hemolytic anemia offers an accessible system for study of the development and regression of an erythropoietic organ. In this species, the spleen is normally a minor organ of erythropoiesis; following bleeding or hemolysis, it becomes a major site of red cell production. The conversion from a predominantly lymphoid organ to an erythropoietic one, and the restoration following recovery of the circulating hematocrit, is an orderly sequence of events of reproducible magnitude. In addition, splenic phagocytosis of phenylhydrazine-damaged erythrocytes occurs concomitantly with erythropoietic development during the period of drug administration and immediately thereafter.

The erythropoietic spleen offers a relatively unused but quite appropriate source for the study of the control of transcription in specialized mammalian cells. In marrow cell populations, an augmented synthesis of ribonucleic acid was observed to be associated with the early stages of erythroid precursor development. This rate declined to low levels in basophilic erythroblasts, although the RNA content of these cells was still greater than at subsequent stages of development. In a comparable manner, the tissue concentration of cytoplasmic RNA increased threefold during the posthemolytic sequence of splenic erythropoiesis; most of this RNA being ribosomal (rRNA). Using an...
in vitro method to determine the capacity for cytoplasmic rRNA synthesis by splenic minces, it was found that the largest increases in the synthesis of cytoplasmic 28S and 18S rRNA were observed with minces derived from animals 2 days before peak enlargement of the spleen and maximal concentration of hemoglobin-containing nucleated spleen cells.\(^6\)

The synthetic rate of cytoplasmic rRNA, however, is not a reflection of transcription alone but includes the post-transcriptional processes of cleavage of rRNA precursors, of association of rRNAs with ribosomal proteins, and of transport of nascent ribosomal subunits from the nucleus to the cytoplasm. Therefore, it was desirable to measure the activity of splenic nuclear RNA polymerase as a function of time following induction of the anemia with phenylhydrazine, so as to follow the transcriptional processes more specifically. To do this, the nuclear polymerases were characterized from control spleens, as well as those in which maximal cytoplasmic rRNA synthesis occurred. The characteristics of splenic nuclear RNA polymerase activity and its fluctuation during the cycle of phagocytosis and erythropoiesis constitute the basis of the present report.

**MATERIALS AND METHODS**

Reagents were obtained as follows: unlabeled nucleoside triphosphates from P-L Biochemicals, Inc. (Milwaukee, Wis.); polyvinylsulfonic acid potassium salt from Eastman Organic Chemicals (Rochester, N.Y.); ribonuclease-free sucrose and actinomycin D from the Mann Research Laboratories (New York, N.Y.); the trisodium salt of phosphoenolpyruvate, Torula RNA, and crystalline rabbit skeletal muscle pyruvate kinase from the Sigma Chemical Company (St. Louis). The labeled substrate \(^{3}\text{H}-5\)-UTP (specific activity, 21 Ci/mM) was purchased from the New England Nuclear Corporation (Boston, Mass.). Nitrocellulose filters, HA type 0.45 \(\mu\), were obtained from the Millipore Filter Corporation (Bedford, Mass.).

**Treatment of Mice**

Male C57Bl/6J mice, weighing 20–30 g, were purchased from the Jackson Memorial Laboratory (Bar Harbor, Maine). The animals were maintained under standard conditions with an intake of a routine diet ad lib. They received three intraperitoneal doses of freshly neutralized phenylhydrazine (0.04 mg/g of body weight), administered at 5 p.m. on the first day and at 9 a.m. and 5 p.m. on the second day.

**Isolation of Nuclei**

A modification of the method of Blobel and Potter was used to obtain nuclei from the murine spleens.\(^7\) Mice were killed by cervical dislocation, and the spleens were quickly removed and placed in cold 0.25 M sucrose-TKM buffer (0.05 M Tris-HCl, pH 7.5, 0.025 M KCl, 0.005 M MgCl\(_2\)) that contained polyvinylsulfate, 10 \(\mu\)g/ml (PVS). Excess moisture was removed by blotting, and the spleens were weighed and minced. The mince was transferred into 8 ml of 0.25 M sucrose-TKM-PVS and was centrifuged at 1500 g at 4°C for 5 min. The supernatant was removed by suction, and 4 volumes of 0.25 M sucrose-TKM-PVS were added to the pellet. The tissue was disrupted with three to four strokes in a Potter-Elvehjem homogenizer, immersed in an ice bath, using a motor-driven Teflon pestle (clearance 0.0095 cm) at 1200 rpm. The homogenate was filtered through six layers of gauze and then was mixed by inversion with 2 volumes of 2.3 M sucrose-TKM-PVS, resulting in a solution with a molarity of 1.62. One volume of 2.0 M sucrose was layered under this, and the interface was allowed to settle for several minutes. The tubes were centrifuged at 38,000 g for 60 min without braking. The supernatant was removed by suction, and material adhering to the wall of the tubes was removed with cotton swabs. The pellet was suspended in 5 ml of 0.25 M sucrose-TKM-
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PVS and was centrifuged at 1500 g for 5 min. After removing the supernatant, the pellet was resuspended in 0.25 M sucrose-TKM without PVS. This suspension was adjusted to a DNA concentration of 1-2 mg/ml for use in the assay of RNA polymerase.

**Assay of Nuclear RNA Polymerase**

The method of Widnell and Tata was modified for the splenic nuclear polymerase. In the absence of (NH₄)₂SO₄, the reaction mixture contained Tris-HCl (pH 8.5), 50 μmoles; NaF, 3 μmoles; 2-mercaptoethanol, 2.4 μmoles, (instead of cysteine); MgCl₂, 2 μmoles; phosphoenolpyruvate, 2 μmoles; pyruvate kinase, 10 μg; GTP, CTP, ATP, 0.3 μmoles of each; nonradioactive UTP, 0.05 μmoles; ³H-5-UTP, 3 μCi; and varying amounts of the nuclear suspension in a final volume of 0.5 ml.

In the presence of (NH₄)₂SO₄, the buffer was Tris-HCl (pH 7.5), 50 μmoles, and MnCl₂, 0.4 μmoles, was substituted for MgCl₂. A saturated solution of (NH₄)₂SO₄, 50 μl, neutralized to pH 7.5 with NH₄OH, was added to achieve a final reaction concentration of 0.4 M. All the other reagents were added in the same amounts as in the assay performed in the absence of (NH₄)₂SO₄. As a consequence of the TKM diluent in the final nuclear suspension, the following components were also present in the reaction mixtures: KCl, 2.5 μmoles; Tris-HCl (pH 7.5), 5 μmoles; and MgCl₂, 0.5 μmoles.

The tubes were incubated at 30°C, and the reaction was stopped at the desired time by chilling in ice and by adding 0.5 ml of cold 10% TCA-0.04 M sodium pyrophosphate. Unlabeled UTP, 0.4 μmoles, and Torula RNA, 0.2 mg, were added at that point, and the mixture was left in an ice bath for 5 min. Four milliliters of 5% TCA-0.02 M sodium pyrophosphate were then added, and the suspension was clarified by centrifugation at 1500 g for 8 min. After the supernatant was discarded, the pellet was washed once and was then suspended in 5 ml of 5% TCA-0.02 M sodium pyrophosphate. The precipitate was collected on a nitrocellulose filter and was washed three times with 5 ml of 5% TCA-0.02 M pyrophosphate. The filters were dried and suspended in 10 ml of a toluene-based scintillation fluor. Radioactivity was determined in an Ansitron counter with an efficiency of 8% for tritium. Nuclear RNA polymerase activity is expressed as pmoles of ³H-UMP incorporated into an acid-insoluble product per milligram of nuclear DNA. DNA content was determined by the method of Burton, and RNA was determined by the method of Schneider.

**RESULTS**

**Chronology of Hematologic Events Following Phenylhydrazine Administration**

In the present study, the chronology was defined as follows: day 0, preinjection control period; days 1–2, period of phenylhydrazine administration; days 3–12, subsequent period of rise and decline of splenic erythropoiesis. The changes in the hematocrit, reticulocyte count, concentration of splenic erythroblasts, and spleen weight have been reported in a previous publication. The hematocrit decreased by nearly 50% between days 1–5. At the same time, the weight of the spleen and concentration of erythroblasts increased and reached their maximum on days 7 and 8, respectively. The peak reticulocytosis was on day 7, and the hematocrit returned to control values on day 8. The spleen weight and erythroblast pool declined to control values by days 11 and 12. These data are consistent with those presented by Coleman when he used the same strain of mice.

**Characterization of Nuclear RNA Polymerase Activity**

The assay conditions for RNA polymerase were developed with nuclei derived from spleens that were removed on days 0 and 5. The latter time was chosen because it was the point of maximal production of cytoplasmic rRNA...
Table 1. Requirements for Nuclear RNA Polymerase Reaction

<table>
<thead>
<tr>
<th>Omissions</th>
<th>Day 0</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>21.0</td>
<td>67.4</td>
</tr>
<tr>
<td>None, temperature 37°C</td>
<td>6.2</td>
<td>43.0</td>
</tr>
<tr>
<td>Buffer</td>
<td>22.0</td>
<td>31.0</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>8.7</td>
<td>56.2</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>1.2</td>
<td>49.5</td>
</tr>
<tr>
<td>ATP, GTP, CTP</td>
<td>2.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Phosphoenolpyruvate, pyruvic kinase</td>
<td>13.7</td>
<td>38.2</td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>None + DNase (50 μg)</td>
<td>17.0</td>
<td>109.0</td>
</tr>
<tr>
<td>ATP</td>
<td>9.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CTP</td>
<td>7.0</td>
<td>10.0</td>
</tr>
<tr>
<td>GTP</td>
<td>11.0</td>
<td>9.0</td>
</tr>
<tr>
<td>None + RNase (230 μg)</td>
<td>0.0</td>
<td>33.0</td>
</tr>
</tbody>
</table>

Incubation was 20 min in duration at 30°C.

following phenylhydrazine administration. The comparison of requirements at these 2 days was necessary to obviate any marked quantitative differences in the requirements of nuclei from control and erythropoietic spleens. The isolation procedure yielded nuclear preparations containing 60% the DNA content of the splenic homogenates. The ratio of RNA/DNA for the nuclei obtained from day 0 spleens was 0.08 and from day 5 spleens was 0.18. The final suspension contained only a rare unbroken cell, and the nuclei were intact and grossly free of cytoplasmic tags when viewed by light microscopy.

Table 1 demonstrates the requirements for UMP incorporation into acid-insoluble material when the assay was run in the absence of (NH₄)₂SO₄. A significant reduction in polymerase activity was observed when the reaction temperature was 37°C, instead of 30°C. This occurred with nuclei obtained at either day 0 or day 5, but the reduction was more marked with the former. The nuclei from day 0 spleens were again much less active in the absence of NaF, although a minor decrease was observed with day 5 nuclei. Similar results were observed in the absence of the ATP-generating system of phosphoenolpyruvate and pyruvic kinase. Although not shown in this table, 2-mercaptoethanol was necessary to achieve a maximal rate of the reaction with nuclei from either source.

The nuclear-dependent incorporation of ³H-UTP into an acid-insoluble product suggested, but did not prove, that an RNA polymerase reaction was measured. The inhibition of the reaction by RNase (Table 1), as well as the solubilization of the radioactive product by alkaline hydrolysis, indicated that the reaction product was a polyribonucleotide. However, this did not distinguish between the enzymatic formation of a heteropolymer or the addition of labeled UMP to existent RNA chains. The data in Table 1 indicate that a marked reduction in enzyme activity occurred when ATP, CTP, or GTP were omitted from the reaction either singly or in combination. Similar results were observed when the reaction was performed in the presence of 0.4 M
(NH₄)₂SO₄. These findings were consistent with formation of a heteropolymer rather than a terminal addition reaction.

The effectiveness of actinomycin D as an inhibitor of the nuclear RNA polymerase reaction is demonstrated in the data of Table 2. Both in the presence and absence of (NH₄)₂SO₄, the nuclei from day 0 and day 5 were markedly inhibited by actinomycin D at a concentration of 1-5 μg/ml. This was consistent with a DNA template-directed polymerase reaction. A further indication that a DNA template was required for the polymerization was that treatment of day 5 nuclei with pancreatic DNase I, 50 μg, resulted in a marked decrease in enzyme activity. In the assay without (NH₄)₂SO₄ a 78% inhibition was observed, while in the presence of 0.16 M (NH₄)₂SO₄, a 34% inhibition was found. Inhibition of enzyme activity DNase I was also seen in day 0 nuclei (Table 1).

Prior studies with nuclei from other tissue sources indicated a marked variation of RNA polymerase activity with changes in the divalent metal concentration, pH, and ionic strength. The effect of these factors was investigated with nuclei from day 0 and day 5. Figure 1 demonstrates the change in polymerase activity as a function of Mg²⁺ or Mn²⁺ concentration. In the absence of additional divalent metal ions, there was virtually no polymerase activity with

![Fig. 1. Effect of divalent cation concentration on splenic nuclear RNA polymerase activity. Details of nuclear isolation and assay of polymerase are described in Methods. Duration of incubation was 10 min. These assays were done in the absence of (NH₄)₂SO₄.](image-url)
day 0 nuclei. With nuclei from day 5 spleens, there was only a modest decrease in the activity compared to that at the optimal Mg$^{2+}$ concentration and approximately a 50% reduction below that observed at the optimal Mn$^{2+}$ level. Aside from the difference between the nuclear polymerase activities in the absence of additional divalent metal ions, the response to increasing concentrations of Mg$^{2+}$ or Mn$^{2+}$ was qualitatively the same. Mn$^{2+}$, between 0.5–2.0 mM, stimulated the polymerase to greater levels than were observed with Mg$^{2+}$. However, at 2–12 mM, the reaction was markedly inhibited by Mn$^{2+}$ but not by Mg$^{2+}$.

Goldberg observed the marked stimulation of rat liver nuclear RNA polymerase with high concentrations of (NH$_4$)$_2$SO$_4$.$^{12}$ This has been corroborated by other workers with nuclei from different tissues and species.$^{13,15,18}$ Other salts have been found to be as effective as (NH$_4$)$_2$SO$_4$, and the observed effect has been attributed to a change in ionic strength rather than that of specific ion. With nuclei from the mouse spleen, a marked increase in polymerase activity was seen to occur at a final (NH$_4$)$_2$SO$_4$ concentration of 0.4 M, and a definite inhibition occurred at higher salt levels. The variation is shown in Fig. 2. Although nuclei from days 0 and 5 spleens demonstrated maximal activity at the same (NH$_4$)$_2$SO$_4$ concentration, those from the control organ were apparently stimulated more at the nonoptimal concentrations. In addition to the increased activity when the ionic strength was elevated, there was difference in the duration of the reaction. At the low ionic strength, the observed extent of the reaction reached a plateau between 15 and 20 min of incubation, while the rate increased up until 45 min of incubation under the condition of high ionic strength.

Another difference between the polymerase activity when assayed at low and high ionic strength, was the pH optimum of the reactions. In the absence of (NH$_4$)$_2$SO$_4$, an optimum at pH 8.5 was observed; while in the presence of the salt, the activity was greater between pH 6.5 and 7.5. This effect of pH, as well
as the response to ionic strength and divalent ion concentration, was quite similar to the observations on nuclei from rat liver.\textsuperscript{13,16}

\textit{Change in Nuclear RNA Polymerase Activity During Splenic Erythropoiesis}

When the polymerase activity was assayed at different times following injection of phenylhydrazine, a striking variation was observed both in the presence and absence of (NH$_4$)$_2$SO$_4$. The results are demonstrated in Fig. 3. In preparations from the control spleens, a mean value of 20 p moles of UMP/mg DNA was incorporated at low ionic strength, and 380 p moles were incorporated in the presence of 0.4 M (NH$_4$)$_2$SO$_4$. These values are similar to those obtained by McGuire and O'Malley\textsuperscript{14} with unstimulated chick oviduct nuclei but are far lower than other workers have found with nuclei from normal rat liver. Even at day 2, a point at which phenylhydrazine was still being administered, there was an increase in nuclear RNA polymerase either at low or high ionic strength. A peak in both the low and high ionic strength activities was observed at day 4. At that point, the activity assayed in the absence of (NH$_4$)$_2$SO$_4$ was ten times greater than the control level and the activity assayed in the presence of (NH$_4$)$_2$SO$_4$ was three times greater than the control levels. A significant decrease in nuclear RNA polymerase activity under both ionic conditions occurred at day 5. This is the time when the rate of synthesis...
of cytoplasmic rRNA had been found to be maximal. The level of the enzyme when assayed at high ionic strength was the same as seen with nuclei from control spleen. In contrast, the low ionic strength activity persisted above the control values until day 8. The fluctuation in nuclear polymerase was similar to the difference in the rate of nuclear RNA synthesis by splenic minces removed at different times following final phenylhydrazine administration.19

DISCUSSION

The ability of the mouse spleen to become a major site of erythrocyte production has been used by investigators to identify some of the molecular events that underlie erythroid development. In light of the morphologic observations of Filmanowicz and Gurney,20 Fujioka studied the in vivo incorporation of 32phosphorus into splenic nucleic acids after erythropoietin administration to hypertransfused polycythemic mice.21 Both RNA and DNA demonstrated increased labeling; the change in the former occurred within 2 hr of injection of the hormone and exceeded the incorporation of the label into DNA for the first 24 hr after injection. Small doses of actinomycin D, when injected simultaneously with erythropoietin, suppressed both the increase in RNA labeling and the induction of an accentuated splenic uptake of 59Fe. Takaku and Ono measured the DNA-dependent nuclear RNA polymerase activities of erythropoietin-treated and control polycythemic mice.22 There was a twofold increase in the high ionic strength activity with nuclei of the hormone-treated animals, while no difference was found between the controls and erythropoietin-treated group when the assays were performed at low ionic strength. Appels and Williams found almost a sixfold increase in DNA-stimulated polymerase activity from immature avian erythrocytes following a phenylhydrazine-induced anemia, as compared to nonanemic controls.23 The enzyme activity was found in the high-speed centrifugal supernatant, probably due to the use of sonication as a means of cell disruption.

To obtain a maximal change in splenic erythroid development, we induced a hemolytic anemia with phenylhydrazine in C57B1/6J mice, a strain with a low rate of endogenous splenic erythropoiesis. The nuclear RNA polymerases obtained from lymphoid-rich and erythroid-rich spleens were similar in response to changes in pH, divalent metal concentrations, and ionic strength. When assayed either at low or high ionic strength, the polymerase activity increased significantly during the period of drug administration and was maximal on day 4. The peak day preceded that of the nadir of the hematocrit decline, as well as the maxima of splenic size. Moreover, the peak specific activities of the heme synthesis enzymes, Δ-aminolevulinic acid dehydratase,11 uroporphyrinogen I synthetase,24 and uroporphyrinogen decarboxylase25 occurred on days 6–7 following the same course of phenylhydrazine treatment in mice. This indicates that the rise in nuclear DNA-dependent RNA polymerase(s) activity is an early event in the development of splenic erythropoiesis.

Finally, the use of a phenylhydrazine-induced hemolytic anemia to stimulate splenic erythropoiesis also leads to marked phagocytosis of damaged erythrocytes by splenic macrophages. The period of accentuated splenic phagocytosis, as observed by light microscopy, was of shorter duration (days 1–3) than was
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the over-all span of splenic erythropoiesis. As transitional increases in human granulocyte RNA synthesis were associated with phagocytosis,26,27 the absolute identification of a rise in nuclear RNA polymerase activity with erythroid development alone must await studies comparable to the present one using erythropoietin-treated hypertransfused or exphypoxic mice. The present study emphasizes the potential of the mouse erythropoietic spleen as a rich source for the purification of DNA-directed RNA polymerase or polymerases associated with erythropoietic development. In this study, the activities of nuclei are compared on the basis of activity per mg nuclear DNA. When conversions are made for tissue DNA concentrations and wet weights,6 the average preanemic spleen had a nuclear RNA polymerase activity of 18 pmoles and 324 pmoles of UMP incorporation per 10 min at low and high ionic strengths, respectively. On day 7 following the start of phenylhydrazine administration, erythroblasts were at least 70% of the total splenic nucleated cells, and there was virtually no detectable phagocytosis.6 At that time, the average spleen contained a low ionic strength activity of 594 pmoles and a high ionic strength activity of 2640 pmoles of UMP incorporated per 10 min. The method of Rutter and Roeder28 has recently been used to solubilize the RNA polymerase or polymerases from nuclei of day 7 spleens in our laboratory. The soluble preparations have a complete dependence on added DNA and will be used for further purification.

REFERENCES

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