RNA Synthesis in Nuclei Isolated from Normal and Friend Virus-infected Mouse Spleen

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SUMMARY

Spleen nuclei were isolated and purified from normal and Friend virus-infected mice. Nuclei from infected spleen were found to contain almost 3 times as much RNA as nuclei from normal spleen. RNA polymerase activity of normal and Friend virus-infected nuclei were compared. Optimal reaction conditions at high and low ionic strength were determined and were found to be similar to normal rat liver nuclei. While the RNA polymerase activity of both normal and Friend virus-infected nuclei was stimulated at high ionic strength, the level of activity of the nuclei from normal spleen reached only 25% of the activity of the infected spleen nuclei. It is suggested that the difference noted in RNA polymerase activity was not simply a result of increased DNA template activity, but rather an increase in the amount of enzyme present in the infected cells.

INTRODUCTION

The neoplastic process initiated by FV is characterized by a proliferation of reticulum cells, splenomegaly, and erythroblastosis. Although the pathological aspects of this disease have been studied extensively (12), the molecular mechanism remains obscure.

Lin and Rich (8) have recently shown that the aggregate RNA polymerase of normal mouse spleen tissue contains only one-eighth the activity of the aggregate enzyme from FV-infected mouse spleen. This enzyme is prepared from a crude homogenate (14); however, advances in the isolation of nuclei makes it feasible to study the RNA polymerase activity of pure and intact nuclei. In this work, we have adapted the procedure of Blobel and Potter (1) to the isolation of nuclei from normal and FV-infected mouse spleen. Furthermore, we have examined chemical composition and biochemical properties of the isolated nuclei in an effort to identify molecular basis for the pathological condition.

MATERIALS AND METHODS

Materials. ATP-14C and ribonuclease-free sucrose were purchased from Schwarz BioResearch, Inc., Orangeburg, N. Y., unlabeled ribonucleoside 5'-triphosphates were from P-L Biochemicals, Milwaukee, Wis., PEP and pyruvate kinase were from Sigma Chemical Co., St. Louis, Mo., and enzyme grade ammonium sulfate was from Mann Research Laboratories.

Animals, Virus, and Tissue. The original FV used in these experiments was obtained from Dr. E. Mirand and has been maintained in DBA/2 mice in our laboratory for 5 years by serial inoculation of 0.2 ml of a 5% cell-free homogenate. Spleens of these mice were used for preparation of nuclei if they were greater than 500 mg and were between 14 and 21 days postinfection.

Analytical Determinations. Diphenylamine determinations for DNA and orcinol determinations for RNA were carried out according to the procedure of Dische (3). Protein determinations were carried out according to the procedure of Lowry et al. (9).

RNA Polymerase. RNA synthesis was assayed in the nuclei by measuring the amount of ATP-14C incorporated into an ice-cold TCA-insoluble residue. The following reaction mixture components were contained in a final volume of 0.5 ml: 2 to 10 X 10^7 nuclei; 0.5 mM CTP, GTP, and UTP; 10 μM ATP-14C (specific activity, 20 mCi/mole); 6 mM PEP; 10 μg pyruvate kinase; and 0.05 M Tris-HCl, pH 8.5. The concentrations of divalent cations and the pH of the buffers were varied according to the experiment. Following incubation for 10 min at 37° the reaction mixture was placed on ice, precipitated with 3 ml of 10% TCA containing 0.04 M sodium pyrophosphate, and centrifuged at 1500 X g for 5 min. The nuclear sediments were resuspended in 3 ml 5% TCA-0.02 M pyrophosphate and centrifuged as before. This was repeated 4 times. Under these conditions, retention of absorbed nucleotide precursors is negligible. The TCA-washed nuclear sediments were dissolved in 1.0 ml of NCS and placed in standard liquid scintillation vials with 15 ml toluene containing 50 mg POPOP and 4.0 g PPO/liter. These samples were counted in a Nuclear-Chicago Mark I liquid scintillation counter at 80% efficiency.

RESULTS

Nuclei were prepared from both normal and FV-infected mouse spleen by a slight modification of the procedure of Blobel and Potter (1) for the isolation of rat liver nuclei. In this

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3The abbreviations used are: FV, Friend virus; PEP, 2-phosphoenolpyruvic acid; TCA, trichloroacetic acid; TKM, 0.05 M Tris-HCl, pH 7.5; 0.025 M KCl; and 0.005 M MgCl₂.

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procedure, the tissue is homogenized in 2 volumes of 0.25 M sucrose-TKM and adjusted with 2.3 M sucrose-TKM to 1.62 M sucrose-TKM. This homogenate is layered on a 2.3 M sucrose-TKM solution and centrifuged at 25,000 rpm in the SW-25 rotor of the Spinco ultracentrifuge. The high concentration of sucrose in the homogenate floats the endoplasmic reticulum and thus prevents its accumulation at the interface of the 2 sucrose solutions.

Slightly better yields of nuclei (50% as opposed to 35%) were obtained in the present work by homogenizing the spleen tissue in 6 volumes instead of 2 volumes of 0.25 M sucrose-TKM buffer. As shown in Fig. 1, A and B, the nuclei are intact and apparently free from cytoplasmic debris. There is some variability in size of nuclei from both sources, which may be a reflection of the different cell types that exist in the spleen. Furthermore, the nuclei from the infected mice appear to be larger than those from the corresponding normal tissue. Other studies confirm this observation (R. Fiel and B. Munson, in preparation). In separate experiments, it was established that dispersed spleen cells were unable to pass through 2.3 M sucrose-TKM solution during centrifugation. A few whole cells were occasionally noted in the nuclei preparations, but these probably represented less than 1% of the total yield.

It has been shown previously that FV-infected mouse spleen tissue contains about 1.5 times as much RNA as corresponding normal spleen on a wet weight basis (10). As shown in Table 1, nuclei isolated from FV-infected spleen contain 2.8 times as much RNA as normal nuclei. The RNA/DNA ratios of normal (0.225) and FV-infected spleen nuclei (0.627) compare to 0.125 for normal rat liver nuclei (1). Likewise, the protein content of the nuclei isolated from the infected tissue is nearly double that of the uninfected spleen nuclei. The protein/DNA ratios of normal and FV-infected nuclei are 3.52 and 6.44, respectively.

The requirements for incorporation of ATP-14C into acid-insoluble nuclear residue at low ionic strength were similar to those presented by Lin and Rich (8). All 4 nucleotides were required for optimal incorporation. A deletion of 1 nucleotide decreased the amount of incorporation by 50%, while deletion of the 3 unlabeled nucleotides decreased incorporation to 40%. Because the nuclei were prepared in a buffer containing Mg2+ ions, incorporation was not completely eliminated when Mg2+ ions were left out of the reaction mixture. In the absence of PEP and pyruvate kinase, only 63% of the optimal activity was obtained because of the relatively high level of nucleotidase activity in both normal and FV-infected spleen nuclei. Mercaptoethanol had little effect upon the RNA polymerase activity, while 100 μM p-chloromercuribenzoate inhibited the reaction. The reaction was also inhibited by actinomycin D, DNase, and RNase. Moreover, RNA synthesis in nuclei isolated from FV-infected mouse spleen was found to

Table 1
Analytical determination of DNA, protein, and RNA in normal and FV-infected mouse spleen nuclei

<table>
<thead>
<tr>
<th>Source of nuclei</th>
<th>DNA</th>
<th>Protein</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (4)</td>
<td>3.52 ± 0.296b</td>
<td>0.225 ± 0.021</td>
<td></td>
</tr>
<tr>
<td>FV-infected (3)a</td>
<td>1.64 ± 1.06</td>
<td>0.627 ± 0.108</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1.83</td>
<td>2.78</td>
<td></td>
</tr>
</tbody>
</table>

a No. of determinations.
bS.D.
Chart 3. The effect on ATP-\(^{14}\)C incorporation into an ice-cold, acid-insoluble product by increasing the pH of the reaction mixture. \(A\), the effect of increasing the pH of the reaction mixture on ATP-\(^{14}\)C incorporation into an acid-insoluble product in the presence of nuclei isolated from normal mouse spleen. \(B\), the effect of increasing the pH of the reaction mixture on ATP-\(^{14}\)C incorporation into an acid-insoluble product in the presence of nuclei isolated from FV-infected mice. \textit{Ordinate}, hundreds of cpm of ATP-\(^{14}\)C incorporated into acid-insoluble product; \textit{abscissa}, pH of the reaction mixture. The pH of the reaction mixture was controlled by adding 25 \(\mu\)moles of Tris-HCl buffer of the proper pH to the reaction mixture. •—•, absence of ammonium sulfate; o— o, presence of 0.4 M ammonium sulfate.

Chart 4. The effect of increasing the concentration of magnesium chloride and manganese chloride on ATP-\(^{14}\)C incorporation into an ice-cold, acid-insoluble product utilizing isolated nuclei from FV-infected mouse spleen. \(A\), absence of ammonium sulfate in the reaction mixture; \(B\), presence of ammonium sulfate in the reaction mixture. •—•, magnesium chloride; •—•, manganese chloride. \textit{Ordinate}, hundreds (A) or thousands (B) of cpm ATP-\(^{14}\)C incorporated into acid-insoluble product; \textit{abscissa}, concentration of divalent cation.

be more sensitive to both actinomycin D and DNase than in nuclei isolated from normal mouse spleen.

The effect of a high concentration of salt on RNA synthesis in isolated mammalian nuclei is well established (15). Chart 1 shows the effect of increasing concentrations of ammonium sulfate on the \textit{in vitro} RNA synthesis in spleen nuclei from FV-infected mice. There is a severalfold stimulation of RNA synthesis at 0.4 M ammonium sulfate followed by a rapid decline in activity to 0.6 M salt and a somewhat slower drop in activity at higher concentrations. The shape of the curve and the level of stimulation is remarkably similar to that reported for rat liver nuclei by Widnell and Tata (15).

Under the conditions of the experiments used in this study, the RNA polymerase activity was directly proportional to FV nuclei concentrations up to \(10 \times 10^7\) nuclei/0.5 ml reaction mixture. This was true at both low and high ionic strength (Chart 2). Similar results were obtained with nuclei from normal mice.

Chart 3, \(A\) and \(B\), shows the effect of pH on RNA synthesis. A well-defined pH optimum was obtained at low ionic strength for nuclei of FV-infected mouse spleen; however, no such optimum was noted for nuclei of normal spleen under the same conditions. It appears rather to be bimodal in nature. A well-defined optimum at pH 8.5 exists for nuclei from both sources at high salt concentrations.

The data presented in Chart 4, \(A\) and \(B\), show the divalent cation dependence of RNA synthesis in nuclei from FV-infected mouse spleen.
RNA Synthesis in Mouse Spleen

The data is similar to that shown by Widnell and Tata (15) for normal rat liver nuclei. The important feature is the comparative stimulation of polymerase activity by manganese chloride and magnesium chloride in the presence and absence of ammonium sulfate and the concentrations of the divalent cations at optimal polymerase activity. At low ionic strength, magnesium is more effective than manganese whereas, at high ionic strength, the situation is reversed.

The differential response of RNA synthesis by isolated nuclei from normal and metabolically stimulated tissue, i.e., normal and regenerating rat liver, at high and low ionic strength, has been shown to be a reflection of template activity (10). To determine whether isolated nuclei from normal and FV-infected mouse spleen respond in a similar manner, we performed the experiment reported in Chart 5. The results show that nuclei from spleen of FV-infected mice are much more active in the synthesis of RNA than nuclei from the spleens of control mice. It can further be seen that nuclei from both sources can be greatly stimulated to synthesize more RNA by increased salt concentrations; however, the nuclei from normal mouse spleen are stimulated much less than the nuclei from infected mouse spleen. The difference in activity of normal and FV-infected spleen nuclei at high ionic strength suggests that something in addition to template availability is responsible for this.

DISCUSSION

The difficulties involved in the isolation of purified nuclei from tumor tissues are well known (4). Fortunately, these problems were not apparent with either the normal or infected spleen tissue used in this study. The only problem encountered during the isolation was that instead of forming a pellet some of the nuclei were trapped at the interphase between the 1.62 M sucrose-TKM homogenate and the 2.3 M sucrose underlay. The Blobel-Potter procedure is based on the principle that endoplasmic reticulum is less dense than 1.62 M sucrose and nuclei are more dense than 2.3 M sucrose. The spleen homogenate, however, also contains a substantial number of whole cells the densities of which apparently fall between that of the 2 sucrose solutions. Consequently, these cells form a matrix into which the nuclei are trapped. Entrapment at the interphase was minimized by using a dilute homogenate which allowed more nuclei to pass into the 2.3 M phase. The nuclei isolated by this procedure were judged to be morphologically intact and free of debris by phase contrast microscopy.

In general, the characteristics of the RNA polymerase reaction utilizing these nuclei were found to be very similar to rat liver nuclei. The data presented here are in excellent agreement with the data reported by Widnell and Tata (15). The effect of high concentrations of monovalent cations as well as the divalent cation requirements assayed at both low and high ionic strength are identical to that reported for rat liver. The effect of divalent cations on the RNA synthetic reaction is somewhat different, however, than that reported by Lin and Rich (8). While the optimal concentrations are the same, magnesium was found to effect more synthesis than manganese in the present work. Although this difference is unexplained, it can be noted that while isolated nuclei were used in this work, Lin and Rich used a soluble “aggregate enzyme” prepared from whole spleen.

The increase in growth seen in the FV-infected spleen cells as well as the increased spleen nuclear RNA content of the infected mice correlates well with the increased in vitro nuclear RNA synthesis. Furthermore, the present data, which show 1.83 times as much protein and 2.78 times as much RNA in the infected nuclei as in the normal nuclei, are consistent with the data presented by Dingman and Sporn (2). These authors have shown that chromatin from metabolically active tissue has a higher total protein and RNA content than the inactive mature avian RBC chromatin.

The molecular perturbations which cause this increased RNA and protein synthesis are not known. It has been shown in other systems that an increase in RNA synthesis can result from an increase in DNA template availability (5, 6, 10, 13).

For example, Pogo et al. (10) have shown this to be the case for the increase in RNA synthesis noted in regenerating rat liver nuclei. One experiment on which this conclusion was based is the differential stimulation of in vitro RNA synthesis by ammonium sulfate of normal and regenerating rat liver nuclei. While nuclei from regenerating rat liver have about twice the capacity for RNA synthesis of nuclei isolated from normal liver, both types of nuclei have the same capacity to synthesize RNA in the presence of ammonium sulfate. The amount synthesized in the presence of the salt was about twice the amount of the regenerating liver nuclei in the absence of the salt. The differential response of nuclei at low and high ionic strength to exogenous bacterial RNA polymerase and the shift of nucleotide composition to a DNA-like base composition also supported this interpretation. Results similar to these have been obtained in normal and hormone-stimulated tissues (5, 6).

The stimulatory response produced by high salt concentration in nuclei isolated from normal spleen is similar to other normal tissues (13). However, the degree of stimulation is far less than has been observed for infected mouse spleen. The stimulation obtained at high ionic strength with the spleen nuclei of FV-infected mice greatly exceeds that obtained from normal nuclei. Therefore, the virus-infected mouse spleen is unlike both regenerating liver nuclei and hormone-stimulated tissue in this respect. This implies that the increased activity of the infected nuclei is not entirely due to an increase in template activity.

There appears to be some difference between the RNA synthetic reaction of nuclei isolated from normal and FV-infected spleen. The pH optimum at low ionic strength is different in that a typical bell-shaped curve is not obtained for the normal nuclei. It may be that there are 2 enzymes in the normal tissue, one which requires magnesium, low ionic strength, and a high pH optimum and a 2nd enzyme which requires manganese, high ionic strength, and a pH optimum of about 8.0 to 8.5. The 2nd enzyme may be preferentially elevated in the infected tissue. This would account for the marked increase in RNA polymerase activity at pH 8.5, when assayed at high ionic strength in the presence of manganese ions. We are attempting to analyze further by solubilizing the
nuclear RNA polymerase and separating different species by density gradient centrifugation by the procedure used for liver nuclei reported by Liao et al. (7).

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. a, isolated nuclei from normal mouse spleen. Acidic orcein, X 430. b, isolated nuclei from FV-infected mouse spleen. Acidic orcein, X 430.
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