Characterization of T7-specific Ribonucleic Acid Polymerase

I. GENERAL PROPERTIES OF THE ENZYMATIC REACTION AND THE TEMPLATE SPECIFICITY OF THE ENZYME

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SUMMARY

The biochemical properties and template specificity of the T7 phage-specific DNA-dependent RNA polymerase have been studied. The enzyme shows the same absolute requirements for DNA, nucleoside triphosphates, and a divalent metal ion as are found for bacterial RNA polymerases, however, the conditions for optimal RNA synthesis are somewhat different. The T7 RNA polymerase initiates T7 RNA chains rapidly with GTP; each enzyme molecule can initiate several T7 RNA chains in the course of the reaction. Hence, an efficient termination mechanism is present in the in vitro system.

Of the helical DNAs tested, only T7 DNA, T3 DNA, and salmon sperm DNA have appreciable template activity with T7 RNA polymerase. A variety of single-stranded and denatured DNAs support RNA synthesis but at a reduced rate. Of the helical synthetic polynucleotides tested only d(I)n, d(C)n, and d(G)n, d(C)n, were active; both support the synthesis of poly(rG). In contrast, both single-stranded poly(d(C)) and poly(d(T)) served as templates. It is suggested that T7 RNA polymerase requires a specific promoter site on DNA for effective transcription; this site is different from that used by bacterial RNA polymerase and may be rich in cytosine and thymidine residues.

The growth of T7 bacteriophage requires the participation of two different DNA dependent RNA polymerases. The bacterial RNA polymerase is required in the initial stages of infection to transcribe the early regions of the T7 genome (1). However, transcription by this enzyme is restricted in its action to a region representing about 20% of the r-strand of the T7 DNA (2). Transcription of the remainder of the T7 genome requires the protein product of T7 gene 1 which is a T7-specific RNA polymerase (3, 4). Purified preparations of the T7 RNA polymerase contain only a single major protein component suggesting that the native enzyme may contain only a single polypeptide chain of molecular weight 107,000. This is smaller and less complex than the bacterial RNA polymerase, which has a molecular weight of 470,000 and which contains at least four different kinds of polypeptide chains (5, 6). Since the T7-specific RNA polymerase appears to carry out efficient site selection for both initiation and termination of T7 RNA in vitro, a study of the properties of the protein and of the enzymatic reaction may cast light on the mechanism of site selection in RNA synthesis. We report here studies of the general properties of the enzymatic reaction carried out by T7 RNA polymerase and on the template specificity of the enzyme.

MATERIALS AND METHODS

Unlabeled nucleoside triphosphates were purchased from Schwarz-Mann (Orangeburg, New York). [α-32P]nucleoside triphosphates were prepared according to Symons (7). [γ-32P]GTP and ATP were prepared by a modification (8) of the method of Glynn and Chapell (9).

DNA was prepared from Escherichia coli phages T2, T3, T5, T7, 213, 3x174, and λ by the method of Thomas and Abelson (10). All phages except T2 were purified by centrifugation followed by sedimentation to equilibrium in a CsCl gradient. DNA from Bacillus subtilis phages β3 and β22 was provided by Dr. Roy Doi, University of California, Davis, Calif., DNA from B. subtilis phase SF50 was provided by Dr. Thomas Trautner (11), and DNA from E. coli phage P2 was obtained from Dr. Richard Calendar, University of California, Berkeley, Calif. DNA from salmon sperm was prepared by the method of Kay el al. (12). E. coli DNA was prepared according to Lehman (13). Strands of T7 DNA were separated according to Summers and Szybalski (14). DNA concentrations are given in terms of total nucleotide concentration.

DNA homopolymers, homopolymer pairs, and alternating co-polymers were prepared as follows: d(T)n·d(C)n (15), d(G)n·d(C)n (16), d(A)n·d(T)n (17), d(A-C)n·d(T-G)n (18), and d(A-T)n (19). RNA homopolymers were purchased from Miles Chemical Company and were prepared as described elsewhere (15).

E. coli RNA polymerase (Fraction 6) was prepared by a modification1 of the method of Berg et al. (20) and had a specific activity of 20,000 to 24,000 dAT units per mg. T7 RNA polymerase preparations (Fraction F) were prepared according to Chamberlin et al. (3), and had specific activities of from 60,000 to 120,000 units per mg.

RNA polymerase assays contained (0.1 ml): 4 µmoles of

1 W. Mangel, personal communication.
concentrations to ensure that a valid assay is obtained. Enzyme assays must be routinely done at two or more enzyme concentrations to minimize this difficulty. Because of the existence of this problem all variation of assay components, however the use of quartz distilled water and freshly prepared reagents seems to prevent or minimize this difficulty. Because of the existence of this problem all enzyme assays must be routinely done at two or more enzyme concentrations to ensure that a valid assay is obtained.

Parameters Affecting Rate of T7 RNA Synthesis

General Requirements—The general requirements for T7 RNA synthesis directed by T7 DNA polymerase are shown in Table I. As expected for a template directed polymerase, RNA synthesis shows an absolute requirement for DNA, the 4 ribonucleoside triphosphates and Mg++. The activity of the enzyme is reduced significantly if a sulphydryl reducing agent such as β-mercaptoethanol is omitted from the reaction. The addition of 10⁻⁴ M p-hydroxymercuribenzoate to the assay system in the absence of β-mercaptoethanol abolished all activity, indicating that the enzyme contains a sulphydryl group necessary for activity. Optimal activity also requires the presence of high concentrations of bovine serum albumin in the reaction as well as in the solution in which the enzyme is diluted. The reason for this requirement is not known, but it may be related to the extreme sensitivity of the enzyme in the ternary RNA polymerase-DNA-RNA complex to inhibition by polyamines (21). Bovine serum albumin is known to have a high binding capacity for a variety of ionic compounds (22). The amount of T7 RNA synthesized in a standard 10-min assay was directly proportional to the amount of T7 RNA polymerase added to the reaction over the range from 0.5 to 50 units of enzyme. A linear relationship between incorporation of labeled nucleotide and the amount of enzyme added to the assay was also obtained with T3 DNA, d(G), d(C), M13 DNA, and salmon sperm DNA. However, occasional assays showed an abnormal response in which little or no incorporation was obtained with amounts of enzyme up to 5 to 10 units, while addition of larger amounts of enzyme gave the expected linear relationship between the amounts of T7 RNA synthesized and the amount of enzyme added in excess of 5 to 10 units. The latter result is that expected if the assay components contain an inhibitor which can inactivate a fixed amount of T7 RNA polymerase. We have not been able to identify the hypothetical inhibitor by systematic variation of assay components, however the use of quartz distilled water and freshly prepared reagents seems to prevent or minimize this difficulty. Because of the existence of this problem all enzyme assays must be routinely done at two or more enzyme concentrations to ensure that a valid assay is obtained.

Kinetics of RNA Synthesis The rate of T7 RNA synthesis is essentially constant for 10 min after initiation of T7 RNA synthesis (Fig. 1). The rate of the reaction then decreases gradually over the next 20 min. Similar results are obtained when T3 DNA, d(G), d(C), M13 DNA, or salmon sperm DNA replace T7 DNA as templates in the reaction, although the actual rate of synthesis with these templates is lower as is discussed below. Measurement of the initial rate of the reaction over the time period from 10 to 60 s (Fig. 1, inset) reveals a slight lag in the initial rate of RNA synthesis followed by a linear rate of nucleotide incorporation into RNA which is equal to that measured in the complete system. In the short-term reaction (inset), reaction mixtures were warmed to 37° for 30 s prior to the addition of T7 RNA polymerase. In the latter experiment [α-³²P]ATP had a specific activity of 2 × 10⁶ cpm per nmole.

### Table I

<table>
<thead>
<tr>
<th>Components</th>
<th>Relative activity</th>
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<tr>
<td>Complete system</td>
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<tr>
<td>-DNA</td>
<td>&lt;0.1</td>
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<tr>
<td>-Mg²⁺</td>
<td>&lt;0.1</td>
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<tr>
<td>-BSAa</td>
<td>33</td>
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<tr>
<td>-GTP, UTP, CTP</td>
<td>&lt;0.1</td>
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<tr>
<td>-β-Mercaptoethanol</td>
<td>71</td>
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</table>

a BSA, bovine serum albumin.

Fig. 1. Kinetics of T7 RNA synthesis by T7 RNA polymerase. RNA polymerase assays were carried out as described under "Materials and Methods" using 90 nmole of T7 DNA and 0.07 μg of Fraction F T7 RNA polymerase. In the short-term reaction (inset), reaction mixtures were warmed to 37° for 30 s prior to the addition of T7 RNA polymerase. In the latter experiment [α-³²P]ATP had a specific activity of 2 × 10⁶ cpm per nmole.
Values obtained for ATP, GTP, UTP, and CTP were 4.7, 16.0, and 6.0, and $8.1 \times 10^{-5} \text{ M}$, respectively. Studies of the initial rate of T7 RNA synthesis at low concentrations of UTP or CTP revealed little or no increase in the lag period during the first few seconds of RNA synthesis at these low concentrations. A slight increase in the lag period was noted with ATP, however, this increase was not sufficient to require a correction in the standard 10-min assay to obtain a true initial rate. However, with GTP concentrations at or below the apparent $K_m$, a large increase in the lag period occurs and hence the value obtained for this nucleotide may reflect a combination of $K_m$ and rate constants for chain initiation and chain elongation. This is probably due to the unique position of GTP at the 5'-terminal nucleoside triphosphate moiety in the RNA chains synthesized by T7 RNA polymerase.

**Temperature**—The rate of T7 RNA synthesis decreases considerably when the assay is carried out at lower temperatures (Fig. 3). The effect of lowered temperatures is greater when T7 DNA is used as template than when single-stranded poly[d(C)] is employed in the temperature range from 10-20°C. This may reflect a restriction on the ability of the enzyme to open the strands of the DNA template at low temperatures, as has been suggested for the bacterial RNA polymerase (25). However, the actual difference obtained between helical and single-stranded templates is not large. Thus, steps in enzymatic RNA synthesis other than strand separation may also exhibit a high temperature coefficient.

**T7 RNA Chain Initiation**

Bacterial RNA polymerase initiates RNA chains in vitro primarily with ATP or GTP as the 5'-terminal moiety (26). With T7 DNA as template both ATP- and GTP-initiated chains are formed by *E. coli* RNA polymerase holoenzyme (8, 27), although it has been suggested that the early T7 mRNA is initiated exclusively with ATP (8). When RNA chain initiation is measured using $[\gamma-32P]$purine nucleoside triphosphates, T7 RNA polymerase is found to incorporate only GTP into T7 RNA (Fig. 4). The incorporation of $[\gamma-32P]$GTP continues for at least 30 min at 37°C with either T7 DNA or d(G)$_n$.d(C)$_n$ as template, although the rate of incorporation is about five times more rapid with the latter template (Fig. 5).

This extended RNA chain initiation could be due to either (1) a very slow and asynchronous rate of RNA chain initiation by different T7 RNA polymerase molecules or (2) rapid recycling of T7 RNA polymerase molecules which initiate and complete a T7 RNA chain and can then reinitiate a new RNA chain. (It should be noted that all experiments measuring incorporation of $[\gamma-32P]$GTP were carried out under conditions of DNA template excess.)

In the case of d(G)$_n$.d(C)$_n$ it is clear that the latter possibility is correct, that is, each T7 RNA polymerase molecule can initiate and terminate many chains. As shown in Fig. 5, 0.08 µg of enzyme, which corresponds to a maximum of 0.75 pmoles of enzyme (assuming a molecular weight of 107,000), has initiated over 1 pmoles of poly[r(G)] chains with GTP in the 1st min and each enzyme has recycled at least 15 times in 20 min. When the kinetics of $[\gamma-32P]$GTP incorporation into poly[r(G)] are studied at very short times, there is no indication of a plateau in incorporation which would result if initiation were complete before termination and reinitiation events began (Fig. 5, inset). Thus, poly[r(G)] chain termination and reinitiation probably begin immediately after synthesis has begun. This could result if there were a high probability of termination by any T7 RNA polymerase molecule during chain growth at any point on the
dG:dC molecule. Alternatively, poly[r(G)] chains might terminate only at the ends of the template molecule. This would still give a class of enzymes which terminate rapidly if chain initiation could take place at any point on the chain; presumably some enzyme molecules would initiate at points near the end of the molecule.

When T7 DNA is used as template for T7 RNA polymerase, there is also a considerable amount of RNA chain termination and reinitiation, although this is not nearly as extensive as with d(G)₉₋₅·d(C)₉₋₅. Thus, 0.08 µg of T7 RNA polymerase has initiated 0.75 pmol of T7 RNA chains, or at least one RNA chain per enzyme in less than 10 min (Fig. 4). Other, less direct evidence also indicates that T7 RNA polymerase molecules can initiate and complete chains quite rapidly. First, RNA chain initiation by T7 RNA polymerase appears to be quite rapid; a constant rate of RNA synthesis is attained within 10 to 15 s after the reaction has begun (Fig. 1). In addition, T7 RNA synthesis becomes resistant to inhibition by ultraviolet-irradiated T7 DNA within 15 s after enzyme is added to the reaction. We show in the following paper that this inhibitor inactivates free T7 RNA polymerase but not enzyme in the ternary DNA-enzyme-RNA complex (21).

If chain initiation by the active T7 polymerase molecules is complete in less than 30 s, then what is the maximum length of time that the enzyme can grow a T7 RNA chain before termination? The r-strand of T7 DNA is about 38,000 nucleotides in length; only about 80% of this length is transcribed by the T7-specific enzyme. Thus, a T7 RNA chain could grow to a maximum of 30,000 nucleotides. We have made preliminary estimates of the chain growth rate of T7 RNA by T7 RNA polymerase which suggest a growth rate of about 100 n per s. At this rate, only 5 min would be required to traverse the entire late region of the T7 r-strand, yet as shown above RNA synthesis continues in vitro for over 20 min. These arguments make it seem very likely that the extended incorporation of [γ-32P]GTP by T7 RNA polymerase is due to a highly efficient termination process which allows reinitiation of many T7 RNA chains by a single T7 RNA polymerase molecule.

When the kinetics of [γ-32P]GTP incorporation into T7 RNA by T7 RNA polymerase are followed during the first few minutes of synthesis, it is clear that the rate of [γ-32P]GTP incorporation is not constant during this first part of the reaction (Fig. 4, inset). There is a period of rapid incorporation of [γ-32P]GTP during the first 30 s of synthesis, followed by a very slow rate of incorporation for 30 s to about 2½ min. The rate of incorporation then increases to an essentially constant rate. We interpret the kinetics in terms of the following model. (a) Essentially all active T7 RNA polymerase molecules initiate an RNA chain in less than 30 s. (b) Many of these chains grow for about 2 to 2½ min and are then terminated at a specific termination site on T7 DNA. (c) T7 RNA polymerase molecules are released and can rapidly initiate another T7 RNA chain. If we assume an RNA chain growth rate of 100 n per s, then this model suggests that there are probably 2 or 3 long transcription units on the r-strand of T7 DNA, each with its own specific initiation-promoter site and specific termination site.

The model does not directly explain the slow continued rate of [γ-32P]GTP incorporation between 30 s and 2 min after the reaction has started. These initiation events could be due to the class of T7 RNA polymerase molecules that initiate chains very slowly. Alternatively, this class of initiation events may be due to enzymes restarting that have already terminated an RNA chain. One might imagine that the current in vitro conditions...
are sufficiently unfavorable that a certain fraction of the enzyme dissociates prematurely before reaching a correct termination signal on the DNA; the growing complex is quite unstable as we show elsewhere (21). A third possibility is that there may be one or more very short transcription units in the late region of T7 DNA which lead to completed chains after a very short period of chain growth.2

If we assume that the amount of [γ-32P]GTP incorporation obtained in about 1 min reflects the number of T7 RNA polymerase molecules able to initiate a T7 RNA chain in the reaction (i.e. that each active enzyme has started a chain but that no extensive reinitiation has occurred), then the fraction of active molecules in the enzyme preparation can be estimated. Assuming that the molecular weight of T7 RNA polymerase is 107,000 (3), then about 20% of the protein in the preparation is active T7 RNA polymerase. Since over 90% of the protein is represented by gene 1 protein as monitored by gel electrophoresis analysis, only about 20% of the T7 RNA polymerase molecules are active. This is a surprisingly small fraction; over 70% of the enzyme in preparations of E. coli RNA polymerase is active by the same criterion (8).

The enhanced incorporation of [γ-32P]GTP with d(G)n-d(C)n template is probably due to a more rapid rate of recycling of T7 RNA polymerase with this template. However, the possibility exists that some of the T7 RNA polymerase molecules in the preparation which are unable to initiate T7 RNA chains are able to initiate chains with d(G)n-d(C)n as template.

2 Studies now in progress (M. Golomb and M. Chamberlin) of the T7 RNA products synthesized by T7 RNA polymerase support the notion that there are several very short transcription units as well as several very long transcription units which are read by T7 RNA polymerase on the T7 genome. Six major RNA products are resolved by electrophoresis in acrylamide-agarose gels. Three of these products are quite short (molecular weight values of about 8.4 x 10^6, 4.4 x 10^6, and 2.0 x 10^6, respectively), while two are quite long (molecular weight values of about 5.6 x 10^6 and 4.5 x 10^6, respectively).

FIG. 6. Effect of template concentration on the rate of RNA synthesis by T7 RNA polymerase. RNA polymerase assays were carried out as described under "Materials and Methods" using 0.08 μg of T7 RNA polymerase Fraction F. With T7 DNA template 100% incorporation corresponds to the incorporation of 2.4 nmoles of AMP; values for other templates are given as percentage of this value.

<table>
<thead>
<tr>
<th>Template</th>
<th>T7 RNA polymerase</th>
<th>E. coli RNA polymerase</th>
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</thead>
<tbody>
<tr>
<td>T7 DNA</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>T3 DNA</td>
<td>52</td>
<td>100</td>
</tr>
<tr>
<td>Salmon sperm DNA</td>
<td>29</td>
<td>23</td>
</tr>
<tr>
<td>E. coli DNA</td>
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</tr>
<tr>
<td>T2 DNA</td>
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</tr>
<tr>
<td>λ DNA</td>
<td>&lt;0.5</td>
<td>45</td>
</tr>
<tr>
<td>T5 DNA</td>
<td>1.5</td>
<td>57</td>
</tr>
<tr>
<td>ψ3 DNA</td>
<td>&lt;0.5</td>
<td>50</td>
</tr>
<tr>
<td>ψ2 DNA</td>
<td>&lt;0.5</td>
<td>27</td>
</tr>
<tr>
<td>SP50 DNA</td>
<td>&lt;0.5</td>
<td>14</td>
</tr>
<tr>
<td>P2 DNA</td>
<td>1</td>
<td>15</td>
</tr>
</tbody>
</table>

Table II
Comparison of template specificities for T7 RNA polymerase and Escherichia coli RNA polymerase with various helical DNAs

RNA polymerase assays were carried out as described under "Materials and Methods" using 0.14 μg of Fraction F E. coli RNA polymerase or 0.54 μg of Fraction 6 E. coli RNA polymerase. From 20 to 80 nmoles of each DNA were added to the assay; where there was significant activity, a saturating amount of polymerase or 0.54 μg of Fraction 6 E. coli RNA polymerase.

Activity of Helical DNA Templates—Bacterial RNA polymerase is able to utilize a variety of helical DNAs as templates for enzymatic RNA synthesis. In contrast, the T7-specific RNA polymerase shows appreciable activity with only a few templates (Table II). Of the phage DNA templates, only T7 DNA and T3 DNA are active (28); appreciable activity is also found with salmon sperm DNA as template. Both E. coli RNA polymerase holoenzyme and T7 RNA polymerase appear to initiate RNA chains at specific sites on the DNA template. Thus, the ability to transcribe a helical DNA signals the presence of one or more specific promoter sites on that DNA. All of the coliphage DNAs tested contain promoter sites for E. coli RNA polymerase, as shown by their ability to selectively initiate early phage mRNA from these templates in vitro (29). In addition, there appear to be promoter sites for the E. coli RNA polymerase on all of the other DNAs tested. However, of the coliphage DNAs tested, only T7 and T3 DNAs contain promoter sites for T7 RNA polymerase, and, in the case of T7 DNA, these sites dictate the synthesis of late T7 phage mRNA (4). Thus, the promoter sequence for T7 RNA polymerase is quite distinct from the promoter sequence recognized by the bacterial RNA polymerase. In addition, the sequence is either long enough that it is not generated by random nucleotide arrangements in E. coli for example, or it is a sequence which is restricted in its use in most genomes.

The amount of DNA required to give a maximal rate of RNA synthesis varied with different DNA templates (Fig. 6). Very low concentrations of M13 DNA were required to saturate the reaction, while saturation with salmon sperm DNA template was not complete even at 2 mM DNA concentration. The DNA saturation curves for T7 DNA and T3 DNA were essentially identical (data not shown) and the saturation curve for T7 DNA was essentially unaltered when the concentration of T7 RNA polymerase in the reaction was reduced 10-fold. The latter result suggests that the requirement for 0.4 mM T7 DNA

3 M. Chamberlin and J. Ring, unpublished observations.
to obtain a maximal rate of RNA synthesis reflects the amount of T7 DNA needed to saturate the T7 RNA polymerase in the initial, template-binding phase of the reaction. We have found that the affinity of T7 RNA polymerase for T7 DNA is much lower than that of E. coli RNA polymerase for the same template (21).

The large differences in the amount of DNA nucleotide needed to obtain a maximal rate of RNA synthesis with M13 DNA and with salmon sperm DNA templates probably reflect large differences in the number of promoter-like sites for T7 RNA polymerase per mole of DNA nucleotide. Thus, a single promoter-like site on the M13 genome (molecular weight \(1.6 \times 10^9\)) would be the equivalent of nine such sites on the T7 DNA r-strand, by this criterion. The very large amount of salmon sperm DNA required suggests that promoter-like sites for T7 RNA polymerase are quite rare on that DNA. An occasional promoter-like site for T7 RNA polymerase might well be generated randomly in the genome of a eucaryotic cell as a result of the great complexity of eucaryotic cell DNA sequences (30).

The activity of T7 RNA polymerase with T3 DNA is about one-half that found with T7 DNA in a standard RNA polymerase assay. However, the assay reflects both the number of active enzyme molecules in the reaction and the relative rates of the four major steps in enzymatic RNA synthesis: template binding, RNA chain initiation, RNA chain growth, and RNA chain termination. In principle, the lower rate of T3 RNA synthesis could result from alteration of any of these separate steps, and in the absence of detailed studies of the individual steps it is difficult to determine the exact source of the difference. It has been suggested that the T7 RNA polymerase may show a lower affinity for the promoter site on T3 DNA (28), which would predict that the lower rate of RNA synthesis for T7 RNA polymerase acting with T3 DNA is due to a lower rate of template binding or perhaps of RNA chain initiation. However, measurements of the rate of T3 RNA synthesis by T7 RNA polymerase during the 1st min of synthesis showed a lag of about 10 to 15 s in attaining a maximal rate of nucleotide incorporation. This is quite comparable to the kinetics of RNA synthesis with T7 DNA as template (Fig. 1), and suggests that the rates of template binding and T3 RNA chain initiation by T7 RNA polymerase are not greatly different from those obtained with T7 DNA as template. At the current time we have not measured the rates of T3 and T7 RNA chain growth nor of the chain termination step by T7 RNA polymerase, and it seems unlikely that the former step would show large differences between T3 DNA and T7 DNA. An alternative possibility to explain the difference in template activity between T3 and T7 DNA with T7 RNA polymerase is that T7 RNA polymerase is heterogeneous; if only 50% of the active T7 RNA polymerase molecules could utilize T3 DNA as template then the observed difference would result. Further studies will be required to distinguish among these different possibilities.

**Table III**

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<tr>
<th>Template</th>
<th>Treatment</th>
<th>RNA polymerase activity</th>
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<tbody>
<tr>
<td>T7 DNA</td>
<td>None</td>
<td>870 ± 1180</td>
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<tr>
<td></td>
<td>10^{-3} μg of DNase</td>
<td>81 ± 230</td>
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<tr>
<td></td>
<td>10^{-4} μg of DNase</td>
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<td></td>
<td>Shearing</td>
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<td></td>
<td></td>
<td>Shearing</td>
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<tr>
<td></td>
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<td>Shearing</td>
</tr>
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</table>

**Activity of Sheared DNA and Helical DNAs Containing Single-strand Breaks**—The activity of salmon sperm DNA as a template for T7 RNA polymerase was interpreted as reflecting the presence of promoter sites for T7 RNA polymerase on that DNA. This would not be unexpected in view of the great complexity of eucaryotic cell DNA. However, it seemed possible that the presence of single-strand or double-strand breaks in the DNA template might enhance the template activity of a DNA which was otherwise inactive. If this were so, then the activity of salmon sperm DNA might simply reflect the fragmented state of the preparation. To determine the effect of single- and double-strand breaks on the template activity of DNA for T7 RNA polymerase, T7, SP50, and T2 DNAs were subjected to shearing and to limited cleavage by pancreatic DNase. While T7 DNA is the homologous template for T7 RNA polymerase, preparations of both T2 DNA and SP50 DNA have shown only traces of activity. Thus, we can study the effects of cleavage on both an active and an inactive template. The results (Table III) show that the introduction of breaks in the DNA template reduces the activity of T7 DNA as template but has little effect on the activity of the nontemplate DNAs. Treatment of 300 nmoles of T7 DNA with 10^{-3} μg of pancreatic DNase under the conditions described is sufficient to introduce about 20 single-strand breaks per T7 genome (31). This reduces the template activity of T7 DNA for both the E. coli RNA polymerase and the T7 polymerase, although the reduction is not large. More extensive treatment with an amount of DNase (10^{-2} μg) estimated to introduce about 150 single-strand breaks per T7 genome reduces the template activity for both RNA polymerases substantially, as does extensive shearing of the template. The activity of the nontemplate DNAs SP50 and T2 is not dramatically altered by these same treatments. There is a decline in the activity with SP50 DNA and a slight enhancement of the activity with T2 DNA, but in neither case is the effect large. While the presence of single-strand breaks may account for the small variations we have observed in the very low activity of DNAs such as T2 (0.5 to 2% of the activity with T7 DNA template), it is unlikely that the activity of
salmon sperm DNA is due entirely to single-strand breaks in the DNA. Thus, T7 RNA polymerase requires the presence of a T7-specific promoter-like sequence in a helical DNA if it is to be efficiently transcribed.

Activity of Denatured and Single-stranded DNA Templates—Denatured T7 DNA is much less active as template for both E. coli RNA polymerase and T7 RNA polymerase (Table IV). However, nearly all denatured or single-stranded DNAs tested are active templates for T7 RNA polymerase as well as E. coli RNA polymerase. The actual activity obtained varies over a wide range with different single-stranded and denatured DNAs. The result suggests that although denaturation eliminates the nearly absolute requirement displayed by T7 RNA polymerase for its promoter site, the presence of regions in various single-stranded DNAs which resembles that site may increase the rate of transcription of those DNAs appreciably. Thus, M13 phage DNA which is a very active template for T7 RNA polymerase may contain a site similar to T7 RNA polymerase recognition site.

Denaturation of T7 DNA leads to a loss of the specificity (3) for transcription of the T7 r-strand by the T7 RNA polymerase. Preparations of both r- and l-strands on T7 DNA were active as templates for the T7 polymerase (Table V), although the small amounts of material available prevented a careful study of the rate of synthesis with each under conditions of DNA excess. The RNA formed in each reaction was tested for its sensitivity to pancreatic RNase, to rule out the possibility that the synthesis detected with r-strand was due to contamination with r-strand to form traces of T7 DNA duplexes. While transcription of helical DNA by RNA polymerases gives rise to single-stranded RNA (32, 33), transcription of single-stranded DNA produces a DNA-RNA hybrid which is resistant to pancreatic RNase (33). Transcription of T7 DNA by T7 RNA polymerase gives RNA which is rendered completely acid soluble by 10 μg per ml of pancreatic RNase, while 79% of the RNA synthesized with single-stranded M13 phage DNA is resistant to this concentration of RNase. The results show that both T7 DNA strands are copied when the single strands are employed as templates. With two different preparations of separated T7 strands, the amount of RNase-resistant product was over 90% for the r-strand preparations and one preparation of l-strands. The other preparation of l-strands gave a lower amount of resistant product and was presumed to be contaminated with T7 duplex material. Thus, ability of T7 RNA polymerase to initiate RNA chains only on the r-strand is lost when the duplex structure of the DNA is disrupted.

Polynucleotides as Templates—Synthetic polynucleotides are highly active as templates for E. coli RNA polymerase. However, the differences between the promoter specificity shown by T7 RNA polymerase and E. coli RNA polymerase are also reflected in the ability of the phage RNA polymerase to use helical polynucleotides as templates (Table VI). While poly[d(A-T)] is highly active as template for the E. coli RNA polymerase, it is extremely poor as a template for the T7 enzyme. Similarly, d(A)ₙ-d(T)ₙ and d(A-G)ₙ-d(T-G)ₙ are efficient templates for the bacterial enzyme but are not used by the phage enzyme. Only d(G)ₙ and d(C)ₙ are active with T7 RNA polymerase and with these templates only the dC-containing strand is transcribed. This result, taken with the specificity of T7 RNA polymerase for initiating T7 RNA chains with GTP may signal a preference by the T7 polymerase for binding to, or initiating RNA chains in dC-rich regions of the DNA. The amount of d(G)ₙ and poly(d(C)ₙ) required to obtain maximal synthesis with 0.05 μg of T7 RNA polymerase in a standard assay was 0.06 mm and 0.003 mm final nucleotide concentration, respectively. These are much lower than the concentrations of T7 DNA required to attain a maximal rate of T7 RNA synthesis (approximately 0.4 mm) and may reflect a high affinity of the enzyme for dC-rich sequences in DNA.

When single-stranded polynucleotides are tested as
templates, there is some loss in the specificity seen with helical templates. Thus, poly[d(T)] is active with T7 RNA polymerase while d(A)n-d(C)n is not. However, in the case of the single-stranded DNAs, the loss in specificity is not complete; while E. coli RNA polymerase uses all of the single-stranded polydeoxyribonucleotides as templates, T7 RNA polymerase is appreciably active only with poly[d(T)] and poly[d(C)].

Polyribonucleotides as Templates—Neither of the two stranded homopolymer pairs r(A)n-r(U)n or r(I)n-r(C)n serves as template for T7 RNA polymerase, although each can be transcribed by the E. coli enzyme. The ability of several single-stranded polyribonucleotides to serve as templates is shown in Table VII. The results are puzzling in view of the template specificity differences seen with helical polymerases. Thus, poly[d(T)] is active with T7 RNA polymerase, although each can be transcribed by the E. coli enzyme. The ability of several single-stranded polyribonucleotides to serve as templates is shown in Table VII. The results are puzzling in view of the template specificity differences seen with helical polymerases.

Unprimed Synthesis of Polyribonucleotides and DNA-dependent Homopolymer Synthesis—E. coli RNA polymerase can carry out two anomalous synthetic reactions. In the absence of DNA, ATP and UTP are utilized to form r(A)n-r(U)n (35), with ITP and CTP are utilized to form poly[r(I-C)] (36). We have not been able to detect formation of labeled polymer by T7 RNA polymerase in the absence of Mn++. However, the reactions of up to 24 hours using labeled ATP, UTP, CTP, or GTP, either alone or in complementary pairs. Conditions employed were 0.1 or 10 μg of enzyme in a standard assay mixture incubated at 37°C.

A second anomalous reaction catalyzed by bacterial RNA polymerase involves DNA-dependent formation of poly[r(A)] (37). The reaction appears to employ stretches of T residues in the DNA to form a long polyribonucleotide chain by “displacement” of single-stranded DNA is usually required. The synthesis of poly[r(G)] with DNAs containing a high fraction of guanine plus cytosine has also been reported (24). In view of the possibility that T7 RNA polymerase favors initiation at C-rich regions of the DNA, it was expected that this enzyme might show an active T7 DNA-dependent synthesis of poly[r(G)].

As shown in Table VIII, traces of DNA-dependent poly[r(A)] synthesis can be obtained with T7 RNA polymerase under conditions which give good poly[r(A)] synthesis with E. coli RNA polymerase. Poly[r(G)] synthesis can be detected in each case as well, but is much lower than that found for poly[r(A)]. Neither poly[r(A)] nor poly[r(G)] synthesis was obtained with native T7 DNA with either E. coli or T7 RNA polymerase.

**DISCUSSION**

T7 RNA polymerase resembles the bacterial RNA polymerases in its general requirements for enzymatic RNA synthesis. However, the reaction conditions which give an optimal rate of RNA synthesis differ for the phage and bacterial RNA polymerases. These differences are probably due to differences in the structure of the two kinds of RNA polymerase; while the bacterial enzyme consists of at least four kinds of polypeptide chain subunits, the phage enzyme contains only a single major component (9).

The template specificity of T7 RNA polymerase with helical DNA.
with a single-stranded template is relative; that is, while a sequence of DNA nucleotides resembling a Class II promoter sequence is still essential, there is more latitude in the nature of the sequence with single-stranded DNA than with helical DNA.

T7 RNA polymerase initiates poly[r(G)] chains rapidly and efficiently with \( d(G)_n \cdot d(C)_m \) or \( d(T)_n \cdot d(C)_m \) as template, but does not utilize any other helical homopolymer pair or copolymer we have tested as template. This suggests that the Class II promoter site may be a dC-rich sequence. Such sequences are known to occur in T7 DNA (14), although no more than 6 C-residues appear in any single, uninterrupted sequence (39). The template activity of single stranded \( d(T)_n \) with T7 RNA polymerase may indicate that the promoter recognition site on T7 RNA polymerase is primarily pyrimidine-specific and only secondarily specific for the 6 amino function (40). Hence, a plausible structure for the Class II promoter sequence would be a dC-rich sequence interrupted by occasional dT residues.

T7 RNA polymerase can initiate many RNA chains per enzyme molecule with both T7 DNA and \( d(G)_n \cdot d(C)_m \) as template, hence, there is efficient termination of T7 RNA and \( d(G)_n \) chains in the enzymatic reaction. In the latter case it is problematical whether or not the enzyme terminates at discrete sites on \( d(G)_n \cdot d(C)_m \) such as single- or double-strand breaks, or whether or not there is simply a high probability of terminating during the growth of a poly[d(G)] chain. In the case of T7 DNA as template, a substantial fraction of T7 RNA polymerase molecules appears to terminate T7 RNA chains only after 2 to 2½ min, suggesting that T7 RNA chain termination is a discrete event directed by a specific termination sequence on the T7 genome. In order to transcribe the entire late region of the T7 genome at a chain growth rate of 100 n per s in 2 to 2½ min, two or three large transcription units would be needed, each with its own Class II promoter site and corresponding termination site.

Rough estimates of the fraction of active molecules in current preparations of T7 RNA polymerase (approximately 20%) suggest that the maximum specific activity of a T7 RNA polymerase preparation in which all enzyme was active would be about 400,000 to 500,000 units per mg. This is quite close to the maximum specific activity (900,000 units per mg) calculated for an enzyme of molecular weight 107,000 which continuously synthesizes T7 RNA at a rate of 100 n per s. If these estimates are correct, they reinforce the idea that template binding, RNA chain initiation, and RNA chain termination are very rapid with the T7 RNA polymerase and that the phage enzyme spends most of its time in the elongation of T7 RNA chains.

REFERENCES


Table VIII

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DNA is quite different from the E. coli RNA polymerase holoenzyme, indicating that the two kinds of RNA polymerase recognize different promoter sequences on DNA. These differences are also reflected in the nature of the RNA transcribed by the two enzymes with T7 DNA as template; while the bacterial RNA polymerase holoenzyme transcribes predominantly regions of the genome coding for early phase functions, T7 RNA polymerase transcribes predominantly late regions (4). It seems convenient to discriminate between the two kinds of promoter sites by designating the promoter site or sites recognized by the bacterial RNA polymerase holoenzyme as Class I promoters and those recognized by the phage-specific enzyme as Class II promoters. While all phage and bacterial DNAs tested contain Class I promoter sites, only T7 DNA, T3 DNA, and possibly M13 DNA contain efficient Class II promoter sites for the T7 RNA polymerase. Class II promoter sites for T7 RNA polymerase are also found on salmon sperm DNA but at a much lower concentration than for T7 DNA.

The specificity of E. coli RNA polymerase for RNA chain initiation at Class I promoter sites is lost when the DNA template is single-stranded or when the DNA helix is broken (38). In contrast, the requirement of T7 RNA polymerase for Class II promoter sites on the DNA template is not lost when the template contains single- or double-strand breaks. The absolute requirement for a Class II promoter site for RNA synthesis by T7 RNA polymerase is lost when the template is single-stranded; however, there are substantial differences between different denatured DNAs or different polydeoxynucleotides as templates. This suggests that the loss of specificity in RNA chain initiation

Table VIII

DNA-directed homopolymer synthesis by Escherichia coli and T7 RNA polymerase

RNA polymerase assays were carried out using the basic assay system, except that the template and nucleoside triphosphates were replaced with those listed. Labeled nucleotides were [α-32P]ATP or [3H]GTP as indicated. Each experiment was carried out at two enzyme concentrations, 0.14 and 0.28 μg of T7 RNA polymerase or 1.1 and 2.2 μg of E. coli RNA polymerase holoenzyme, to ensure that the incorporation measured was directly proportional to the amount of enzyme added. The results are expressed as the average of the two determinations corrected to the smaller amount of enzyme in each case. The amount of DNA template used was: T7 DNA, 60 nmoles; M13 DNA, 20 nmoles; salmon sperm DNA, 80 nmoles.
Michael Chamberlin and Janet Ring


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