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Regulation of promoter selection by the bacteriophage T7 RNA polymerase in vitro

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ABSTRACT

During bacteriophage T7 infection a phage-specified RNA polymerase transcribes the late phage genes in two temporal classes (class II and class III). In this report, we show that the purified phage polymerase discriminates between the class II and class III promoters in vitro as a function of variables that alter the stability of the DNA helix. These variables include ionic strength, temperature, and the presence of denaturing agents such as dimethyl sulfoxide. In general, initiation at the class II promoters is preferentially inhibited as helix stability is increased. Conditions required for the establishment of salt-resistant transcription complexes by the T7 RNA polymerase have been determined; the establishment of stable complexes at the class II promoters requires the synthesis of a longer nascent RNA transcript than does formation of such complexes at the class III promoters. A comparison of the nucleotide sequences of several class II and class III promoters suggests certain features that may be responsible for the different responses of these promoters to helix destabilization. The conservation of structural features that are peculiar to the class II or class III promoters indicates that these features are important in regulation of T7 transcription in vivo. Experiments which bear on the physiological significance of these features are discussed.

INTRODUCTION

During infection, the late genes of bacteriophage T7 are transcribed by an RNA polymerase that is specified by one of the early phage genes (1,2,3). Transcription of the late genes is regulated and proceeds in two overlapping temporal classes: the class II genes are transcribed from 6 until 15 min after infection, the class III genes from 6-8 min after infection until lysis (4,5). The only T7 gene which has been found to exert control over late transcription (other than gene 1, which encodes the RNA polymerase) is gene 3.5, which specifies an endolysin (4,6). Expression of gene 3.5 is required for the release of newly synthesized phage DNA from a membrane bound state (6), and it has been reported that expression of this gene may also result in alterations in the selective permeability of the cell membrane (7).

Unlike the host enzyme, which is structurally complex, the T7 RNA

polymerase consists of a single protein species that is capable of initiation and termination *in vitro* in the absence any auxiliary factors (1,3). Due to its structural simplicity the T7 RNA polymerase offers an attractive system with which to study polymerase/promoter interactions. In this report, we show that utilization of the class II and class III promoters by the T7 RNA polymerase *in vitro* is differentially affected by variables that alter the stability of the DNA helix. These variables include ionic strength, temperature, and the presence of denaturing agents such as dimethyl sulfoxide (DMSO). Comparison of the nucleotide sequences of a number of promoters for the T7 RNA polymerase suggests certains features that may be responsible for the different responses of the class II and class III promoters to helix destabilization *in vitro*, and which may be important in the regulation of T7 transcription *in vivo*.

MATERIALS AND METHODS

Synthesis of RNA and analysis by gel electrophoresis. Unless otherwise indicated all reactions contained in a volume of 50 l: 8mM $MgCl_2$; 40 mM Tris-HCl, pH 7.9; 5mM dithiothreitol (DTT); 0.1mM EDTA-Na_4; 4 mM spermidine-HCl; 5% (v/v) glycerol; 0.4 mM ATP, GTP and CTP; 0.1 mM α-^{32}P-UTP (specific activity of 160 µCi/µmole); 2.5 µg of T7 DNA, and 10 units of T7 RNA polymerase (3). At the conclusion of the incubation period (usually 10 min), unlabeled UTP was added to a concentration of 1 mM and incubation was continued for an additional 3 min. Reactions were stopped by the addition of an equal volume of: 2% (w/v) SDS, 50 mM EDTA-Na_4 and 100 µg/ml yeast RNA.

Where indicated, 5 µl portions of the stopped reactions were spotted onto 2.5 cm diameter 3MM (Whatman) filter paper discs. The filters were batch washed three times in 10% TCA, 10 mM NaPPi, twice in acetone, dried, and counted in a toluene-based scintillation fluid.

For gel analysis of high molecular weight RNA, nucleic acids were precipitated at -20° by the addition of KCl (0.15M) and 2.5 volumes of ethanol. The RNA pellets were dried *in vacuo*, taken up in 20 µl of electrophoresis buffer (1.1 M formaldehyde; 0.1 M $NaPO_4$, pH 7.0; 0.1% SDS; 2mM-EDTA-Na_4) and heated at 65° for 5 min (8). After cooling, glycerol (5%) and bromophenol blue were added to the samples which were then loaded into slots in a 3mm thick 0.8% agarose gel cast in the horizontal apparatus described by Studier (9). Gel and running buffer were as described above. Electrophoresis was carried out at 50 V for 6.5 hrs. The

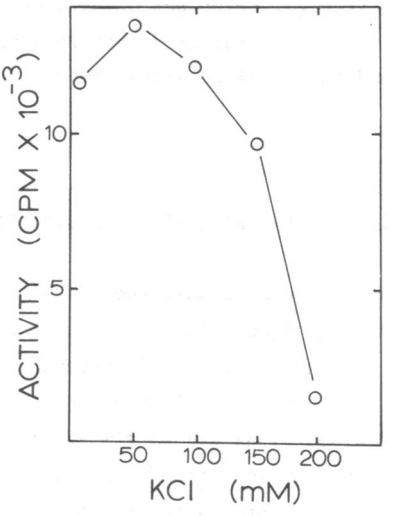
gels were dried on a Hoeffer gel dryer set at 55°, and exposed to X-ray film. For the analysis of low molecular weight RNA, the pellets were taken up in cracking buffer and run in 4% polyacrylamide gels as described by Studier (10).

RESULTS

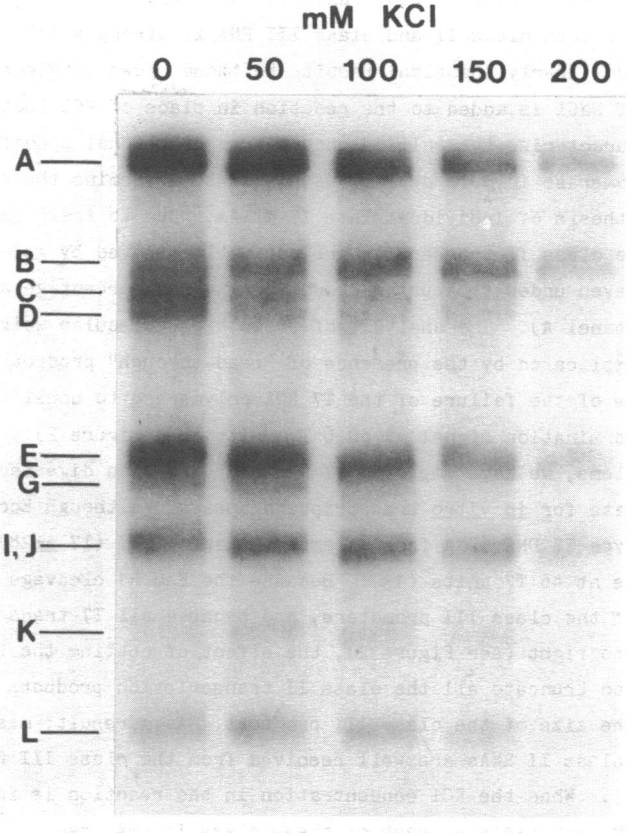
Effect of ionic conditions on the activity and specificity of the T7 RNA polymerase.

It had been previously reported that the T7 RNA polymerase transcribes the class II genes poorly *in vitro* (11,12,29), but that class II transcription could be enhanced by lowering the Mg^{++} concentration from 20 mM ("standard conditions", see reference 3) to a range between 5 and 10 mM (4). The inhibition of class II RNA synthesis at higher ionic strengths is not limited to Mg^{++}; as the concentration of KCl in the reaction is increased from 0 to 150 mM, a preferential inhibition of class II RNA synthesis is observed (Figures 1 and 2). Note that above 150 mM KCl, synthesis of both class II and class III RNA is strongly inhibited (Figure 1, panel A). Nearly identical results to those shown in Figure 1 are obtained if NaCl is added to the reaction in place of KCl (data not shown).

To characterize the salt-dependent transcriptional specificity of the T7 RNA polymerase in more detail, we wished to determine the effect of KCl on the synthesis of individual late T7 mRNAs. Due to their large sizes, most of the class II transcripts are not well resolved by gel electrophoresis, even under denaturing conditions in low percentage agarose gels (Figure 3 panel A). The analysis of these high molecular weight RNAs is further complicated by the presence of "read-through" products which are a consequence of the failure of the T7 RNA polymerase to consistently recognize the termination signal at 60.6 T7 units (see Figure 2). To circumvent these problems, we have employed T7 DNA that has been digested with Eco R1 as a template for *in vitro* transcription assays. Although Eco R1 does not cut wild-type T7 DNA, DNA from an *amber* mutant of T7 (T7 *am*28) is cut by this enzyme at 46 T7 units (13). Because the Eco R1 cleavage site is to the left of the class III promoters, and because all T7 transcription is from left to right (see Figure 2), the effect of cutting the template with Eco R1 is to truncate all the class II transcription products without altering the size of the class III products. As a result, most of the shortened class II RNAs are well resolved from the class III RNAs (Figure 3, panel B). When the KCl concentration in the reaction is increased from 0 to 150 mM, synthesis of each of these class II RNA species is prefer-


ACTIVITY (CPM X 10^{-3})
10
5
50
100
150
200
KCl (mM)

a


mM KCl
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b

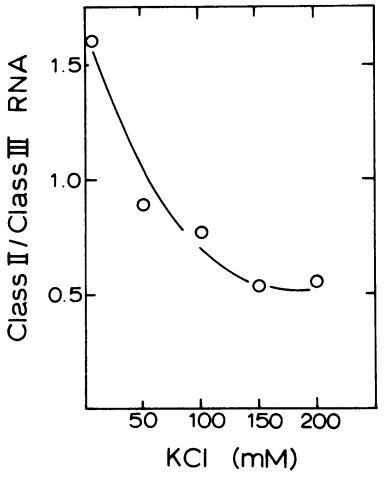

Figure 1. Effect of KCl on the activity and specificity of the T7 RNA polymerase. Reactions containing KCl as indicated were incubated at 37° for 10 min. Panel A: The amount of RNA synthesized (measured as ^{32}P-UMP incorporated) is plotted as a function of the KCl concentration. Panel B: RNA was isolated from the samples by phenol extraction and hybridized to Hpa I restriction fragments of T7 DNA immobilized on nitrocellulose filters (26). The positions of the Hpa I DNA fragments are indicated by letters to the left of the autoradiograms. Panel C: The data in Panel B were analyzed by densitometry. The amount of RNA hybridizing to Hpa I fragments C and D (which arise from the class II region, see Figure 2) relative to that hybridizing to fragment B (from the class III region) is plotted as a function of the concentration of KCl.

entially inhibited. In contrast, all of the class III RNA species continue to be made in the presence of 150 mM KCl.

KCl inhibits the T7 RNA polymerase before initiation.

If high levels of KCl (200 mM) are added to the reaction shortly before, or at the same time as the substrate triphosphates, synthesis of RNA is almost completely blocked (Table 1, and see Figure 1). In contrast, if KCl is added shortly after initiation the same spectrum of RNA products is completed as in the absence of KCl (Figure 4, lanes 12 and 13). By determining the sizes of the products at intervals after intiation, we have found that the chain elongation rate for the T7 RNA polymerase is 160-170 nucleotides/sec at 30° and that this rate of elongation is not affected by the addition of KCl shortly after initiation (data not shown). From these experiments we conclude that the effects of KCl on T7 transcription are exerted prior to or during initiation, and that once transcription is

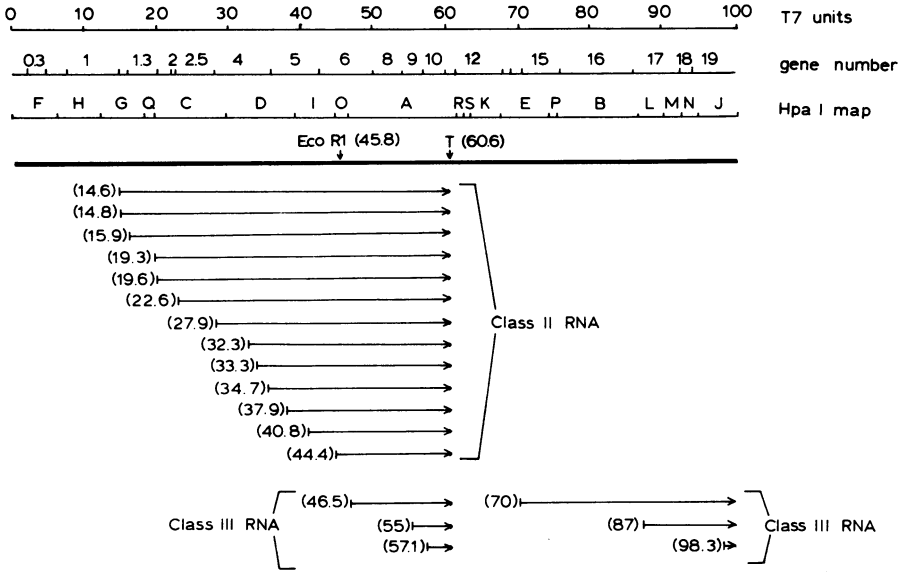

Figure 2. Transcription map of bacteriophage T7. The T7 genes are identified by gene number and grouped according to their function and observed time of expression (27). Distances from the left end of the genome are given as percentage of total genome length (T7 units); the alignment of the genetic and physical maps is derived from a variety of sources (9,28, Studier, personal communication). The position of the Eco R1 cleavage site (13) and the termination signal (T) for the T7 RNA polymerase (Rosa, personal communication) are indicated above the heavy line. At the bottom of the figure, the RNA products synthesized by the T7 RNA polymerase *in vitro* are represented by arrows. The number in parentheses before each arrow denotes the promoter location for each transcript in T7 units (11,12,21,23,29-33 and Carter and McAllister, in preparation). The promoters at 38, 41, and 44 units are weak, and do not give rise to visible bands in the gels presented in Fig. 3.

underway the transcription complexes are resistant to KCl.

Formation of salt-resistant complexes by the T7 RNA polymerase.

The results described above suggest that ternary complexes consisting of RNA polymerase, template DNA, and nascent RNA chain should be resistant to levels of KCl that prevent initiation by the free enzyme. To detect these salt resistant complexes, and to determine whether the conditions for their formation differ at the class II and class III promoters, enzyme and template were incubated together in the presence of an incomplete mixture of ribonucleoside triphosphates and then challenged with 200 mM KCl. Simultaneously with the KCl challenge, the remaining substrate

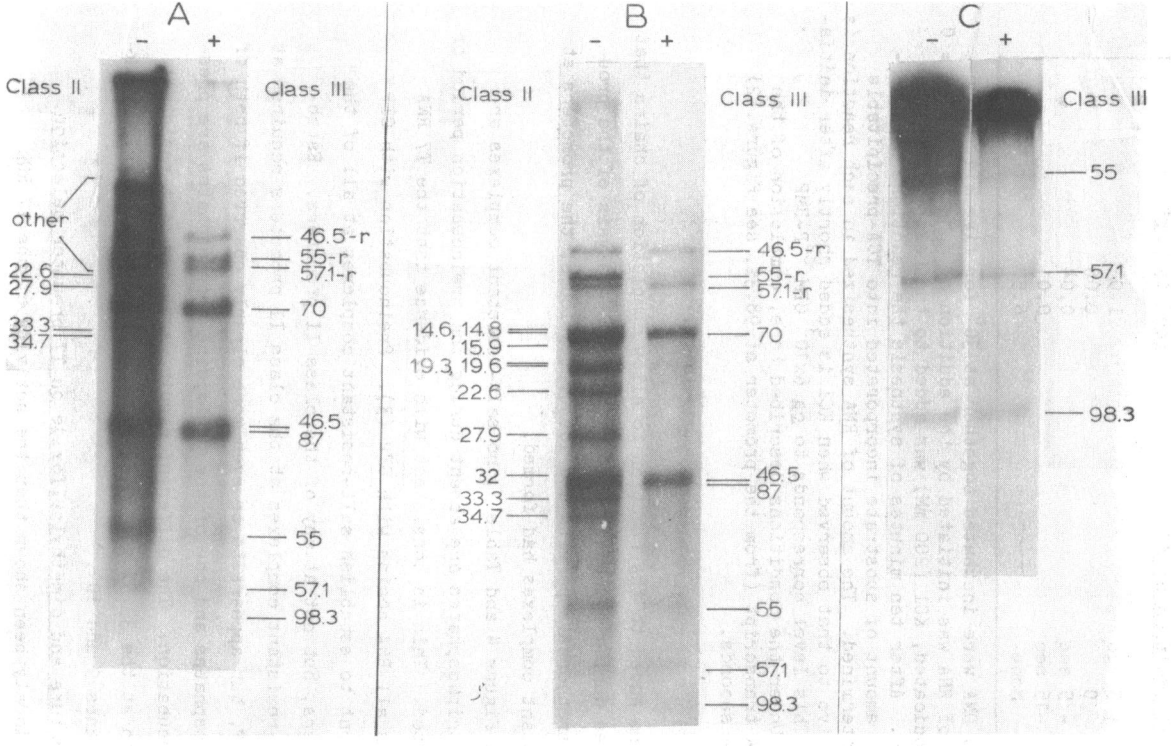

Figure 3. Effect of KCl on the synthesis of individual late T7 RNA species.
RNAs transcribed in the presence (+) or absence (-) of 150 mM KCl were analyzed by electrophoresis through 0.8% agarose gels under denaturing conditions (panels A and B) or through 4% polyacrylamide gels under non-denaturing conditions (Panel C). Template DNA was as follows: Panels A and C, wild-type T7 DNA; Panel B, Eco R1-digested am 28 DNA. Individual RNA species are identified in the margin of the figure by a number which corresponds to their promoter location; "read-through" products which result from failure of the RNA polymerase to terminate at 60.6 T7 units are designated by their promoter location followed by the suffix "-r" (see Figure 2).

Table I. Effect of time of addition of KCl on RNA synthesis by the T7 RNA polymerase

Time of addition of KCl	Relative RNA synthesis by ten minutes*
+ 5 sec	1.0
0	0.06
- 5 sec	0.04
-15 sec	0.01
none	6.5

Enzyme and DNA were incubated together at 30° for five min, whereupon synthesis of RNA was initiated by the addition of substrate (time = 0 sec). Where indicated, KCl (200 mM) was added to the reactions at the times indicated. After ten minutes of synthesis the reactions were terminated and the amount of substrate incorporated into TCA-precipitable material was determined. The amount of RNA synthesized in each reaction is expressed relative to that observed when KCl is added shortly after initiation (5 sec). This level corresponds to ca $6x10^3$ CPM ^{32}P-UMP incorporated. Under the conditions described here, completion of the smallest T7 RNA transcript (from the promoter at 98.3%, see Figure. 2) would require 4 seconds.

triphosphates were added to the reaction to allow completion of chains that had been initiated during the preincubation period. Analysis of the products by gel electrophoresis permits the identification of the promoters at which salt-resistant complexes had formed.

As shown in Figure 4 and Table 1, no salt-resistant complexes are formed when all triphosphates are absent during the preincubation period or when GTP is omitted. This is consistent with evidence that the T7 RNA polymerase starts all RNA chains with GTP (3). Preincubation with GTP alone is sufficient to establish salt-resistant complexes at all of the class III promoters, but not at any of the class II promoters. Establishment of salt-resistant complexes at the class II promoters requires at least GTP and ATP, but appears to be even more clearly observed if both of the purine triphosphates and one of the pyrimidine triphosphates are present during preincubation. The different requirements for salt-resistant complex formation at the 33.3% and 34.7% promoters are particularly striking (Figure 4, lanes 9 and 10).

Effect of temperature and dimethylsulfoxide on T7 promoter selection.

It had previously been shown that the activity of the T7 RNA polymerase exhibits a strict temperature dependence, and that the activity curve is paralleled by the temperature dependence of tight binding of the enzyme to T7 DNA (33). The ability of the polymerase to form salt-

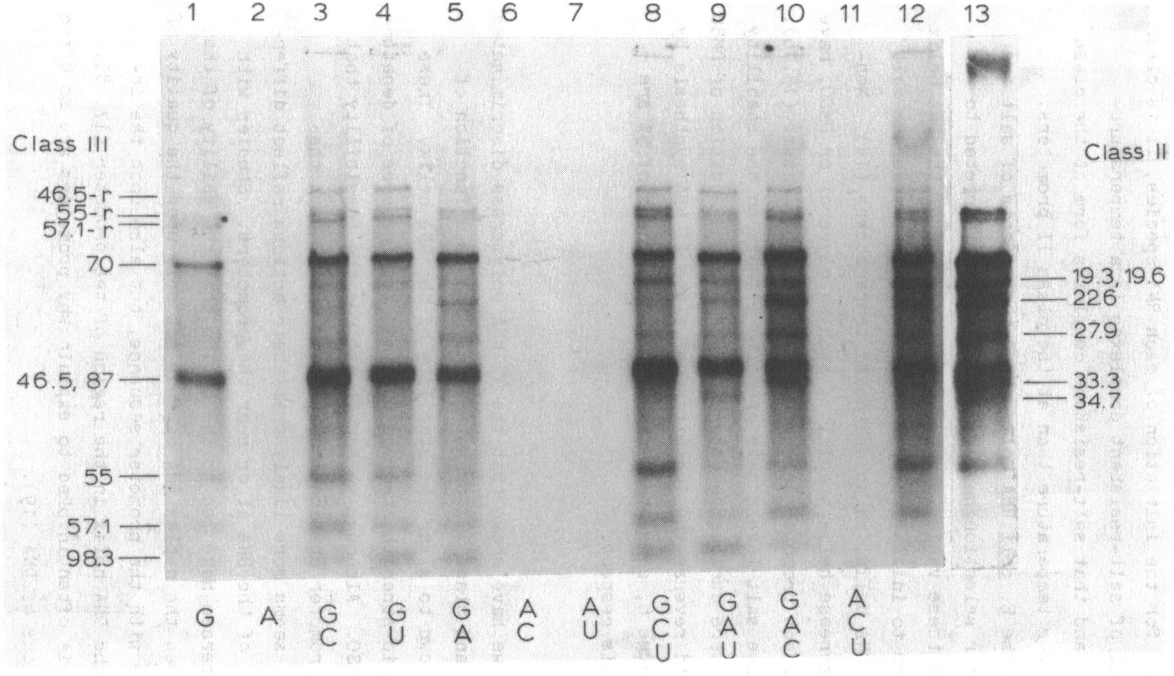

Figure 4. Formation of salt-resistant transcription complexes at the class II and class III promoters. RNA polymerase and Eco-R1 digested T7 DNA were incubated together at 30° in the presence of an incomplete mixture of ribonucleoside triphosphates as indicated (G=GTP, C=CTP, A=ATP, U=UTP). After 5 min the remaining substrate triphosphates and sufficient KCl to give a final concentration of 200mM were added simultaneously. Incubation was continued for an additional 10 min and the samples were analyzed by electrophoresis in 0.8% agarose gels. In the reaction presented in Lane 12 no triphosphates were present during the preincubation period and the addition of KCl was delayed until 5 sec after the addition of a complete substrate mixture. In Lane 13 the products synthesized in the absence of KCl are presented. RNA species are identified by promoter location as described in Figure 3.

resistant complexes at the class II and class III promoters as a function of temperature was determined as described in Figure 5. Although the high background in the autoradiogram prevents an accurate estimation of the transition temperature for the initiation of each RNA species, it is clear that the establishment of salt-resistant complexes is a temperature-dependent phenomenon, and that salt-resistant complexes form at the class III promoters at a lower temperature than at the class II promoters.

In studies with the E. coli RNA polymerase, the effects of salt and temperature on promoter selection have generally been interpreted to reflect the effect of these variables on the melting open of the DNA helix that occurs just prior to initiation, for it is known that moderate salt levels stabilize the DNA helix, as do decreased temperatures (14). Non-ionic agents which decrease helix stability, such as glycerol or DMSO, have also been shown to affect promoter selection by the E. coli enzyme (15,16). Since DMSO and moderate salt levels have opposite effects on the stability of the DNA helix, we were curious to determine whether the addition of DMSO to the T7 system could reverse the inhibition of class II RNA synthesis by KCl. As shown in Figure 6, concentrations of DMSO in excess of 5% are quite effective in this respect.

DISCUSSION

In this report, we have shown that the T7 RNA polymerase discriminates between the class II and class III promoters in vitro as a function of variables that are known to alter the stability of the DNA helix. These variables include ionic strength, temperature, and the presence of denaturing agents such as DMSO. Although we cannot exclude the possibility that these agents affect promoter utilization by their action upon the polymerase itself, it seems more likely that their actions reflect difference in the structure of the DNA at or near the promoters. Studies with the E. coli RNA polymerase have led to the notion that the ability of the polymerase to melt open the duplex DNA depends not only upon the quality of fit of the polymerase with the promoter sequence, but also upon the inherent stability of the DNA helix in the region of the promoter (17,18). The latter principle is often invoked to explain why promoters are so often found in AT-rich regions of DNA (19).

The nucleotide sequences of promoters for the T7 RNA polymerase for which data are currently available are presented in Table 2. Class II promoters are in the upper part of the Table, class III promoters in the

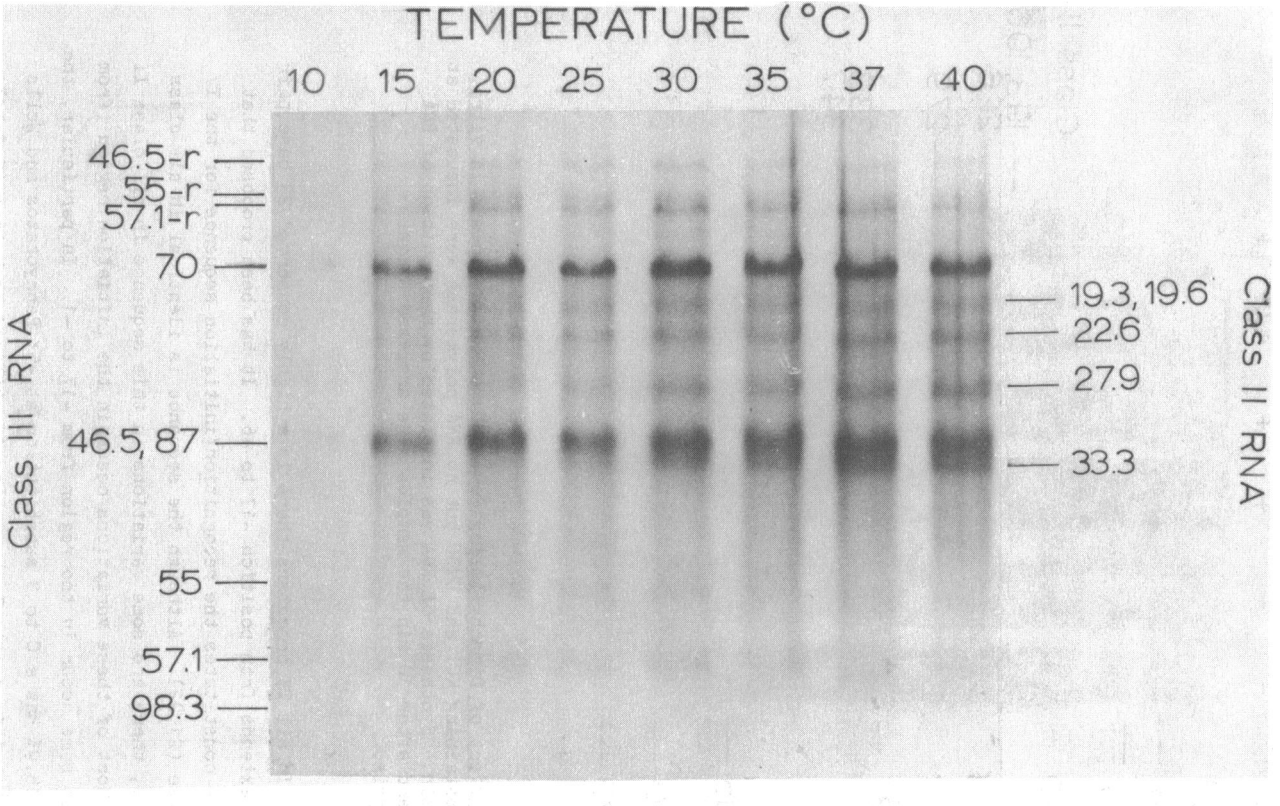

Figure 5. Effect of temperature on promoter selection by the T7 RNA polymerase.
RNA polymerase and T7 DNA were incubated at the indicated temperatures in the presence of an incomplete mixture of substrate triphosphates (GTP, ATP and CTP). After 5 min, KCl was added to a final concentration of 200 mM, the missing triphosphates were added, and the reactions were shifted to 30°. After 10 min, the products were prepared for analysis by gel electrophoresis.

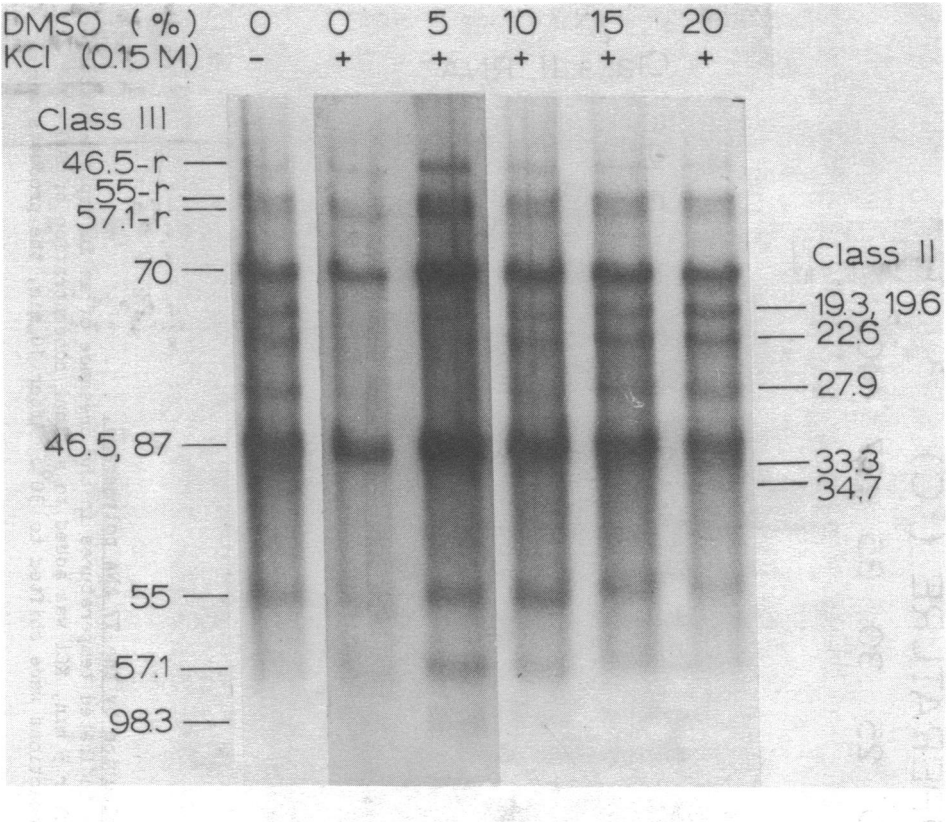

Figure 6. Effect of Dimethylsulfoxide (DMSO) on RNA synthesis in vitro. Reactions containing KCl and DMSO but no RNA polymerase were incubated at 30°C for 5 min. Synthesis of RNA was initiated by the addition of RNA polymerase and after 10 min the samples were analyzed by gel electrophoresis.

lower part. In all T7 promoters there is a highly conserved 23 base pair region that extends from position -17 to +6. It has been proposed that this sequence constitutes the recognition/initiation sequence for the T7 RNA polymerase (21,22). Although the sequence is identical in all class III promoters, there are some variations in this sequence in all class II promoters. Most of these variations occur in the initiation region (from +1 to +6) but some occur in the region from -17 to -1. In particular, the promoter at 15.9% has a C to G substitution at -5; Panayotatos and Wells have noted that this particular promoter is utilized much less (5-fold) efficiently than either the 14.6% or 14.8% promoters, and they have sug-

TABLE II. STRUCTURE OF PROMOTERS FOR THE T7 RNA POLYMERASE.

PROMOTER CLASS	PROMOTER LOCATION	-35 -30 -25 -20 -15 -10 -5 +1 +5 +10 +15 +20 +25	REFERENCE
II	(14.6)	5'-.....TCGCGTTCGCGTAACGCCAAATCAATACGACTCACTATAGAGGGACAAACTCAAGGTCATTCGCAA.....-3'	(33)
II	(14.8)	TTATGATTGACCTTCTTCCGGTTAATACGACTCACTATAGGAGAACCTTAAGGTTTAACTTTAAGACCCTTA	(23)
II	(15.9)	GACCAGTTGAAGGACTGGAAGTAATACGACTCAGTATAGGGACAATGCTTAAGGTCGCTCTCTAG	(23, Dunn, pers. commun.)
II	(19.3)	CGTGTGGAGTATAGTTAACTGGTAATACGACTCACTAAAGGAGGTACACACCATGATGTACTTAAT	(32)
II	(19.6)	TGATATGCCAGATGGTCACGCTTAATACGACTCACTAAAGGAGACACTATATGTTTCGACTTCATT	(32)
II	(33.3)	GTTTCGCTGGAGAGTCCCATTCTAATACGACTCACTAAAGGAGACACACCATGTTCAAACTGATTA	(McAllister and Carter, in prep.)
II	(34.7)	TAAGTGGTGGCCTTCATGAATACTATTCGACTCACTATAGGAGATATTACCATGCGTGACCTAA	(McAllister and Carter, in prep.)
III	(46.5)	GATAAACTCAAGGTCCCTAAATTAATACGACTCACTATAGGGAGATAGGGCCTT	(21)
III	(55)	CGGAATTTAATACGACTCACTATAGGGAGACCTCATCTT	(21)
III	(57.1)	AACTTCTGATAGACTTCGAAATTAATACGACTCACTATAGGGAGACCACAACGG	(21)
III	(70)	TGTGGTGGCTCGAAATTAATACGACTCACTATAGGGAGAACAATACGACACGGGAGGGTT	(33; Rosa, pers. commun.)
III	(87)	AACTTAGGGAGCGTAGGAAATAATACGACTCACTATAGGGAGAGGCGAAATAA	(21)
III	(98.3)	TTACTGAAAAGACACGATAAATTAATACGACTCACTATAGGGAGAGGAGGGACGAAAGGTTACTATATA	(Stahl and Siebenlist, pers. commun.)

Table II. Structure of promoters for the T7 RNA polymerase.

For simplicity, only the sequence of the non-transcribed DNA strand is presented. Since all transcription in T7 is from left to right (34,35) the orientation presented is the same as that in the genome. Nucleotides are numbered relative to the site where RNA synthesis is initiated (+1). The upper part of the table presents the nucleotide sequences of class II promoters, the lower part those of class III promoters. The source of information for each promoter sequence is indicated to its right. The sequence of the promoter at 70% published by Oakely et al (33) is incorrect from position -16 to -32. The correct sequence, derived by M. Rosa (personal communication) is presented in this table. The start sites for RNA synthesis at the 14.6, 46.5, 55, 57, 70, and 87% promoters have been confirmed by 5' sequence analysis of the RNA products (21,33), start sites in the remaining promoters are by analogy to these locations. A number of structural features of the promoter sequences are highlighted in the table as follows. The length of the unbroken A/T stretch extending leftward from position -13 is underlined. The highly conserved region from -17 to +6 is boxed in, and nucleotides that deviate from the conserved sequence are indicated by an asterisk (*). A Hinf I recognition sequence (GANTC) is present in all T7 promoters in the region from -11 to -7.

gested that this may be due to the importance of this nucleotide in promoter recognition (22,23). Other class II promoters deviate from the conserved sequence at positions -2, -13 and -16 but appear to be effectively utilized *in vitro* (Figure 3). Perhaps the quality of polymerase: DNA contacts at these positions is less critical than at -4.

The class II and class III promoters differ strikingly in the region extending leftward from position -12. Whereas in all class III promoters there is a run of AT pairs that extends unbroken for 8-11 nucleotides, in class II promoters the AT sequence is interrupted by a GC pair after only 4-6 nucleotides. In some cases the AT sequence is interrupted by only 1 GC pair (the 14.6% promoter) while in others no AT pairs are observed for another 4 positions (the 14.8% promoter). The effect of AT pairs on local helix destabilization has not been adequately measured, but the estimates of Tinoco *et al* (24) suggest that the differences observed between the class II and class III promoters in the region from -13 to -21 could weaken the double helix in this region by free energy changes of 1.4 to 6.2 kcal. Recently, Strothkamp *et al* (20) have proposed a model for promoter selection by the T7 RNA polymerase in which the enzyme binds non-specifically to double stranded DNA and moves along the DNA in a process of diffusion. During this migration the probability of melting open of regions along the helix is increased. When a specific promoter sequence is encountered, the polymerase "melts-in" to form a binary complex of greatly increased lifetime. The AT rich sequence from -12 leftward may affect either the formation or the stability of this binary complex.

Regions of DNA to the left of -21 are probably not required for initiation, as a HpaII-TaqI fragment of the 14.8% promoter which lacks this region is still transcribed with characteristic efficiency *in vitro* (22). The contribution of the region to the left of -21 on salt or temperature dependent promoter utilization has not been directly determined. However, we have observed that class II or class III promoters inserted into the Bam H1 site of pBR322 are utilized with the same kinetics in infected cells as their counterparts in the intact T7 genome, even when flanked by as little as 60 base pairs of authentic T7 DNA (McAllister and Morris, in preparation).

A consideration of the structure of the class II and class III promoters in the "initiation-region" from +1 to +6, together with the data of Figure 4, suggests that the class II and class III promoters differ markedly in their requirements for the formation of salt resistant complexes.

Preincubation with GTP alone would be expected to result in the synthesis of a trinucleotide at all class III promoters, and this appears to be sufficient for the formation of salt-resistant complexes at these promoters. In contrast, efficient formation of salt-resistant complexes at the class II promoters (for example the 33.3 and 34.7% promoters) appears to require the presence of GTP, ATP and one of the pyrimidine triphosphates, conditions that would result in the synthesis of at least a sexanucleotide at these promoters (Figures 4 and 7). Strothkamp et al (20) have noted that there are sites downstream from the initiation site at which the polymerase "pauses" during elongation and that modified (alkylated) enzymes which are incapable of polymerization become arrested at certain of these sites. It is possible that a conformational change resulting in the formation of a highly-stable ternary complex occurs at these sites. If so, it will be of interest to determine whether the distance from the initiation site to the pause site differs for the class II and class III promoters.

As a result of the discussion above, we propose that T7 promoters have at least three functional, partially overlapping domains. First, there is a highly conserved "recognition" sequence that extends from -17 to -1. Some positions within this region (e.g., -4) may be more important than others (e.g., -2, -3, -16) in the quality of recognition by the polymerase. To the left of the recognition sequence and overlapping it (-23, to -13) there is an AT rich "melting" region that may be important in destabilization of the DNA helix at the promoter. Differences in this region appear to correlate with the salt and temperature dependent utilization of the two classes of promoter. There is a moderate level of sequence conservation among the class III promoters in this region. To the right of the recognition sequence is the "initiation region" (+1 to +6). The sequence of this region is highly conserved among the class III promoters, but divergent among the class II promoters. The significance of the structure of this region on promoter utilization is not clear, but class II promoters appear to require a longer nascent RNA transcript to establish stable polymerization complexes then do class III promoters.

The availability of many of the class II and class III promoters on recombinant plasmids will permit a more detailed study of the salt, temperature and initiation requirements of each of these promoters. Furthermore, the presence of a Hinf I site in all T7 promoters (Table 2) should permit the construction of hybrid (class II/class III) promoter sequences which will facilitate future studies of the relationship between

the structure and function of these promoters.

Physiological significance.

During infection, the class II and class III regions of T7 DNA are transcribed at different times and in different amounts. We had previously noted that in cells infected with phage that are defective in gene 3.5 the switch from class II RNA synthesis to predominately class III RNA synthesis does not occur (4). The product of gene 3.5 is an endolysin; expression of this gene is required to release nascent phage DNA from a membrane bound site and may result in marked alterations in the selective permeability of the cell membrane (6,7). The different responses of the class II and class III promoters to conditions *in vitro* may reflect the responses of these promoters to a changing local environment in the infected cell as a result of gene 3.5 expression. De Wyngaert and Hinkle (25) have recently reported that late T7 RNA synthesis is sensitive to inhibitors of DNA gyrase *in vivo*, and have suggested that unwinding of the DNA helix near the late promoters may be required for their efficient utilization. It will be interesting to determine whether the synthesis of class II and class III RNAs is differentially affected by these drugs.

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