N4 virion DNA dependent-RNA polymerase: initiation sequences utilized by the enzyme on heterologous templates

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ABSTRACT

Bacteriophage N4 virion-encapsulated RNA polymerase, the enzyme responsible for transcription of the phage early RNAs, is unable to use duplex linear DNA as a template. In contrast to other RNA polymerases, the enzyme transcribes denatured N4 DNA with *in vivo* specificity. The promoter sequences for three sites of transcription initiation on the N4 genome have been determined and found to contain conserved sequences and two sets of inverted repeats. In order to define the minimal sequence requirements for N4 virion RNA polymerase activity, we have screened several heterologous DNAs, amounting to 64,328 bases, for their ability to support transcription. Several sequences allowing specific initiation were found. Their location, properties and the relation to N4 virion RNA polymerase promoters are discussed.

INTRODUCTION

Bacteriophage N4 is a double-stranded DNA virus specific for *E. coli* K-12 strains, unique in regulating transcription through the activity of three distinct RNA polymerases, two of which are N4-coded (1,2). The first of these, the N4-virion RNA polymerase, is injected into the host along with the viral genome at the start of the life cycle and is responsible for the synthesis of early phage RNAs (3). This enzyme is composed of one polypeptide of molecular weight 320,000 (4). *In vitro*, the purified virion RNA polymerase is unable to use duplex N4 DNA as a template. Unlike other DNA-dependent RNA polymerases, the enzyme recognizes its promoters on single-stranded DNA (7). Double-stranded DNAs are only utilized if the template is supercoiled and *E. coli* single-stranded DNA binding protein (SSB) is present (5). Accordingly, active *E. coli* DNA gyrase and SSB are required for *in vivo* transcription by the enzyme (5,6).

The promoter sequences of three sites of initiation for the N4 virion RNA polymerase have been determined and found to contain both conserved sequences and two sets of inverted repeats (7). It is of interest to determine which, if any, of the conserved sequences and inverted repeats are required for promoter function. Unfortunately, the small number of natural N4 virion polymerase

Figure 1. Sequence of N4 virion RNA polymerase promoters (7). Non-coding strands are shown, written in a 5' - 3' direction. Conserved sequence regions are boxed, and inverted repeats are marked by arrows.

promoters makes it difficult to draw conclusions from the consensus sequence (Figure 1). A larger set of sequences, similar to the natural N4 promoters, but displaying relative enhanced or reduced promoter activity relative to wild type could help establish significant features of promoter sequence. One way to generate such a set is through site-directed mutagenesis of existing promoters. Any portion of the promoter-sequence whose alteration disrupts promoter activity is probably essential to function. Site-directed mutagenesis studies, however, examine only a small portion of the total possible sequences. In theory, a sequence bearing little resemblance to the starting promoter, but, nevertheless, highly active, could exist. A site-directed study would never uncover a promoter of this type. Additionally, such studies define only those alterations necessary to inactivate a promoter by a very few base changes. They do not necessarily define minimal requirements for promoter activity.

An alternative approach was taken in order to define minimal promoter sequences recognized by the N4 virion RNA polymerase. Several heterologous DNAs amounting to 64,328 bases were screened for virion RNA polymerase activity. Since the N4 virion RNA polymerase promoters encompass an 18 bp region of conserved sequences and inverted repeats, the probability of finding a close match to a sequence of such complexity is low. Therefore, it was our expectation that this approach would define minimal requirements for specific initiation. Several sequences allowing specific initiation by the virion polymerase were found. Their location, properties, and relation to *in vivo* virion polymerase promoter sequences are discussed below.

MATERIALS AND METHODS

Enzymes and Chemicals

Restriction enzymes BamHI, EcoRI, RsaI, HinfI, TaqI, HaeIII, and DdeI were purchased from BRL. S1 nuclease, RNA sequencing enzymes and polynucleotide kinase were from P-L Biochemicals. ³²P-labeled radionucleotides were from

Amersham. The N4 virion RNA polymerase was purified from N4 virions as has been described previously (4). SV40 DNA and a pBR-322 clone containing the complete genome of SV40 were a gift of Robert G. Martin (NIH). Bovine papilloma virus (BPV) DNA was isolated from a pBR-322 clone, a gift from Peter Howley (NIH). Clones of the relevant portions of bacteriophage T7 were obtained from William Studier (Brookhaven National Labs). ØX174 RF form I was obtained from B. Strauss of this Department. M13 mp7 DNA was obtained from BRL, and grown in *E. coli* strain JM 103 as described in the company's M13 cloning/sequencing manual. pBR-K, a pBR322 clone containing the N4 HpaI-K fragment with two in vivo and in vitro sites of initiation for the virion polymerase (7,8,9), was purified from *E. coli* strain W3350 as described by Maniatis et al. (10).

In vitro RNA Polymerase Assay

The conditions used for in vitro RNA synthesis were similar to those described previously (4). Final concentrations were 10 mM Tris-HCl, pH8, 10 mM MgCl₂, 1 mM EDTA, and 1 mM DTT. Glycerol was omitted. All nucleotides except GTP were at 1 mM. GTP varied between 1-100 µM, depending on the desired specific activity. The concentration of DNA restriction fragments varied (0.5-5 µg) depending on the strength of the promoter assayed. Virion RNA polymerase (700 µ/mg) was present at 0.1-5 units (4). Reactions were initiated by addition of polymerase and 1-10 µCi of [α -³²P] GTP to the remaining components at a final volume of 50-100 µl, and incubated 10 minutes at 37° C. At this point, GTP was added to bring the concentration up to 1 mM, and the reactions were incubated for an additional 20 minutes at 37° C. This procedure ensured elongation of initiated RNA. When end-labeled RNA was required for RNA sequencing or for promoter strength comparison, 100-1000 µCi[γ -³²P]-GTP, synthesized by the method of Johnson and Walseth (11), were added. Reaction products were ethanol precipitated, dissolved in formamide-dye mix, and resolved on 8M urea-polyacrylamide gels (10).

Southern Hybridization of In vitro RNAs Synthesized by the Virion RNA Polymerase

DNA was restricted with the appropriate restriction enzyme, and electrophoresed through nondenaturing 5% acrylamide gels (10). Procedures for capillary Southern transfer to Zeta-Probe (Bio Rad) membranes were followed as per the manufacturer's recommendations. Blots were probed either with total ³²P RNA, or with specific bands detected on gels by autoradiography. In the latter case, the gel slice containing the labeled RNA was added directly to the hybridization container, where it eluted by diffusion. Hybridization was in 1x

Denhardt's at 65°C, overnight. Blots hybridized to RNA from total reactions were washed with 6x, 3x and 1x SSC for 30 min at 65°C, while blots hybridized to gel slice RNA were washed only twice with 3x SSC. Blots were dried and exposed at -70°C with intensifying screens.

Procedure for Determining of Sequences Recognized by the N4 Virion RNA Polymerase on Restriction Fragments from non-N4 DNAs

Due to the low activity of the polymerase on many of the templates tested, several methods were required to localize the sites of initiation. On non-N4 templates, only a small proportion of the transcripts produced by the virion polymerase in vitro are full-length, which makes assay by "runoff" transcription difficult. Two procedures were used to overcome this problem. When the strength of these promoters was 2-10% that of a wild type N4 promoter, a combination of S1 nuclease mapping (12) and 5' enzymatic RNA sequencing (13) was used. S1 studies took advantage of the stable DNA/RNA hybrid formed in vitro during transcription of single-stranded templates by the virion polymerase (6). To form the DNA/RNA hybrid, 10-50 µg of DNA was digested with one of several restriction enzymes and electrophoresed on nondenaturing acrylamide gels to resolve fragments which were recovered by electroelution. The purified restriction fragments were denatured and directly used as templates in a standard virion RNA polymerase assay using [$\alpha^{32}\text{P}$]-GTP. Reactions were terminated by addition of S1 buffer (12) and S1 (50-220µ). Samples were incubated with S1 at room temperature for 1-60 min and terminated by the addition of 2.5 volumes of ethanol. After precipitation and resuspension in 95% formamide/dye mix, samples were electrophoresed through urea/polyacrylamide gels and the labeled species were located by autoradiography. The above approach allowed rapid screening for restriction fragments containing virion polymerase promoters.

Since strand separation was not performed in the above experiments, the initiation site determined is restricted to two possible regions, one on each strand of the fragment used. To unambiguously determine the initiating nucleotides, 5' RNA sequencing was then performed. Since the N4-virion RNA polymerase initiates only with GTP, [$\gamma^{32}\text{P}$]-GTP was added directly to a virion polymerase assay containing the appropriate restriction fragment. Reactions were incubated for 10 min, and unlabeled GTP was then added to allow elongation of the [$\gamma^{32}\text{P}$]-GTP-initiated RNAs. Up to 1 mCi of [$\gamma^{32}\text{P}$]-GTP per reaction was sometimes necessary to achieve adequate incorporation. After synthesis the reaction mix was passed through Sephadex G-50 minicolumns (14), ethanol

precipitated and electrophoresed through urea/polyacrylamide gels. The 5' end-labeled RNAs were located by autoradiography, excised, and eluted by diffusion into 100 μ l phenol-saturated, 10 mM Tris-HCl, pH 8, 1 mM EDTA overnight. After ethanol precipitation and resuspension, RNA sequencing was performed with ribonuclease T1 (for G), ribonuclease U₂ (for A), *B. cereus* ribonuclease (for A + C) and Phy M ribonuclease (for U + A) (13). 20% or 25% polyacrylamide-7M urea gels were used to resolve the 5' sequences (7).

When the strength of the initiation site was too low to catalyze the synthesis of sufficient labeled RNA for sequencing, a different approach was used. After 5' end labeling of the DNA fragment, the strands were separated by polyacrylamide gel electrophoresis (10). Each strand was used as a template for run-off transcription, as described above, followed by S1 nuclease digestion. Samples were electrophoresed next to G+A and C+T sequencing ladders generated by Maxam-Gilbert sequencing (15) of the DNA fragment tested.

Sequence of Initiation Sites

In order to determine the sequences around each initiation site, the published sequences of M13mp7 (16), pBR322 (17), ϕ X 174 (18), T7 (19), Bovine Papilloma virus (20) and SV40 (21) were consulted. When a 5' RNA sequence was available, it was tested by computer against the entire template to ensure it defined a unique site of initiation. The total pBR322 sequence was also tested against the consensus of natural N4 virion polymerase promoters to determine whether any inactive sequence had a higher percent homology to the natural promoters than those active in the polymerase assays.

Promoter strengths were compared through hybridization of [γ^{32} P]-GTP labeled RNA to Southern blots of the same templates. The template was restricted with an enzyme separating identified promoters. N4/Xba-F, a restriction fragment of N4 containing two in vivo virion polymerase promoters (7), was added as a control. [γ^{32} P]-GTP (0.5 mCi) was combined with unlabeled GTP to a final concentration of 50 μ M, and enough template was added to the standard assay to saturate the enzyme. Incubation was for 30 min. The reaction mixture was treated with DNase I (40 units) and RNasin (120 units, Promega Biotec) to remove template DNA. The [32 P]-GTP initiated RNA was purified away from unreacted GTP and used to probe Southern blots of the template with the same restriction digest. After hybridization, washing, and visualization by autoradiography, the appropriate regions of the blots were removed for counting. Only relative strengths were determined.

Figure 2. Transcripts produced by N4 virion RNA polymerase on various denatured or single-stranded templates. Standard RNA polymerase assay conditions allowing maximum [α - 32 P]GTP incorporation were used (Materials and Methods). A 10 min pulse of 5 μ Ci [α - 32 P]GTP per reaction was followed by a 20 min chase with 1 mM cold GTP. SV40, pBR322, and F-episome DNA were restricted with *Hinf*I prior to denaturation. 10 μ g total DNA was present in each assay. Products were electrophoresed on 7% polyacrylamide 8M urea gels. Sizes of RNAs are approximate.

RESULTS

Virion Polymerase Activity on Non-N4 Templates

Initially, various DNAs were restricted, denatured, and added to a standard assay containing the virion polymerase. The templates were chosen for screening on the basis of the availability and the existence of published DNA sequences. One N4 restriction fragment, consisting of the last 1100 bp of *Hpa*I-K (9), was also tested. As previously noted (4), these non-N4 templates are poor substrates for the virion RNA polymerase relative to denatured N4 DNA. If reaction products are run on gels, however, discrete RNA species are observed for all templates, as shown in Figure 2. Although total incorporation is less than 1-2% that of an equivalent amount of N4 DNA, the small number of discrete RNAs observed indicates that specific initiation is taking place. Since the templates compared are all rather small (<5000 nucleotides in length), it can be calculated that the promoter sites allowing synthesis of

Figure 3. RNA synthesis on denatured pBR322 restricted with *Hinf*I. 10 μ g of pBR322 DNA was used under standard virion RNA polymerase reaction conditions. The 130 n band detected after electrophoresis (A) was eluted and hybridized as described in Materials and Methods to *Hinf*I-restricted pBR322 nitrocellulose blots (B), (C) hybridization of nick-translated pBR322 DNA to the same *Hinf*I-restricted pBR322 nitrocellulose blots.

these transcripts cannot share more than 30% similarity with natural N4 promoters.

Total reaction products were hybridized to Southern blots containing the restricted DNA that was used in each transcription reaction (not shown). Hybridization to only some restriction fragments of each template was observed, indicating that weak promoters are not frequent enough to be distributed through all the restriction fragments of each template. Moreover, if unique RNA species, such as the 130 n transcript made when *Hinf*I-digested pBR322 is used as template are excised from the gel and hybridized to Southern blots, specific hybridization to a single restriction fragment occurred (Figure 3). This further demonstrates that the observed RNAs probably arise from unique initiation sites.

Determination of Sites of Initiation by S1 Mapping and RNA Sequencing

Figure 4 shows S1 mapping of the sites of transcription initiation for some of the DNAs tested using the DNA/RNA hybrid which is the product of transcription by the virion polymerase on single-stranded templates (6). Since

Figure 4 S1 mapping of sites of initiation of RNA synthesis by virion RNA polymerase on SV40 RsaI 294 bp (A) and 226 bp (B) restriction fragments. 50 μ g DNA was restricted, 5' end-labeled using polynucleotide kinase, electrophoresed and recovered as described in Materials and Methods. The purified fragments were denatured and used as a template for N4 virion RNA polymerase synthesis using unlabeled ribonucleoside triphosphate. S1 digestion and electrophoresis of products was as described in Materials and Methods. Arrows indicate initial fragments and S1-protected regions. 1: DNA template; 2: no RNA synthesis, 60 min S1 treatment; 3: RNA synthesis, 10 min S1 treatment; 4: RNA synthesis, 60 min S1 treatment. At the bottom of the figure, the position of the S1 result in both strands of the tested fragment are indicated. Since both strands are present, the strand used as template is not determined by this experiment.

the DNA/RNA hybrid is formed directly during transcription the presence of nontemplate strand has little effect. Most fragments showed one, or at most, two S1-protected products.

Those fragments giving the strongest signals in the S1 assay were subsequently used as templates for synthesis of labeled RNA for 5' end sequencing. Figure 5 shows representative results for two RNA species synthesized by the virion polymerase on pBR322. The sequences shown were readily aligned with those published for pBR322 (17) at position (-) 1691 and (+) 1570. Computer search showed no other plausible matches. [γ - 32 P] labeled RNA was isolated from reactions supported by other templates, but the quantity

Figure 5. RNA sequencing of the two major RNAs produced from pBR322 *Hinf*I 506 bp template by the virion RNA polymerase. [γ - 32 P]GTP RNA produced by the N4 virion RNA polymerase on denatured template was electrophoresed, the RNA was eluted, concentrated, and sequenced as described in Materials and Methods.

was not sufficient for unambiguous identification of the initiation site by 5'-end sequencing.

Figure 6 presents three *in vitro* transcription initiation sites identified by S1 mapping on the separated strands of two T7 TaqI restriction fragments. Two initiation sites are present in T7 TaqI-84. Although not yielding as precise information as sequencing of the 5' ends of the RNAs, this technique allows for an approximate location of the position of initiation when RNA was produced in insufficient quantities for sequencing. The G closest to the 3' end of the S1 product has been designated in these cases as +1 since the N4 virion RNA polymerase initiates only with GTP (4).

Table 1 summarizes the positions of all promoters whose initiation sites

Figure 6. S1 mapping of virion RNA polymerase initiation sites on strand-separated restriction fragments (A) T7 TaqI-84; (B) T7 TaqI-515. Conditions as in Materials and Methods. 1: DNA template 2: no RNA synthesis, S1 treated; 3: RNA synthesis, 4: C+T; 5: A+G. Arrows indicate S1 products.

were determined by the above procedures. The promoter regions are scattered fairly randomly throughout the DNA molecules. There does not seem to be significant clustering in intergenic regions, or utilization of known promoters for other polymerases (prokaryotic or eukaryotic) on these templates.

Relative Promoter Strengths

Relative promoter strengths within each template molecule were determined by probing blots containing the restricted template with [$\gamma^{32}\text{P}$] GTP RNA synthesized by the polymerase, using the same restricted template. The restriction digest was chosen to isolate already identified initiation sites on separate fragments. An example of the results is seen in Figure 7. Plasmid DNAs pBR322 or pBR(K), which carry N4 promoters P1 and P2, were restricted with *Hinf*I, denatured and used as templates for run off transcription in the presence of [$\gamma^{32}\text{P}$ -GTP]. Reactions were carried out at template excess and therefore, the amount of [$\gamma^{32}\text{P}$] initiated RNA hybridizing to a given restriction fragment is a rough measure of the ability of a promoter to catalyze binding and RNA synthesis. Under these conditions, only transcription originating from fragments containing P1 (721), P2 (230) and the initiation site present in the 1630 bp pBR322 *Hinf*I fragment is detected. Quantitation

Table 1
Position of N4 virion RNA polymerase initiation
sites on heterologous templates

Designation	Template	+1 position
HinfI 154	pBR322	+909
HinfI 221	pBR322	-1375
HinfI 506-1	pBR322	-1691
HinfI 506-2	pBR322	+1570
HinfI 1630	pBR322	+330
RsaI 472-1	ØX174	+1843 or -1493
RsaI 472-2	ØX174	+1525 or -1751
TaqI 84-1	T7	+36434
TaqI 84-2	T7	+36452
TaqI 515	T7	-36015
Hae III 340	BPV	-1907
RsaI 226	SV40	+3993 or -3951
RsaI 294	SV40	+3661 or -3625

revealed that this initiation site is 200 fold weaker than any of the N4 wild type promoters. No promoters are found with activity equal or near equal to natural promoters.

DISCUSSION

This study was motivated by the assumption that initiation specificity by the N4 virion RNA polymerase could be uncoupled from initiation efficiency. Since *in vivo* promoters for *E. coli* RNA polymerase vary over several orders of magnitude in initiation efficiency, this is not unwarranted. The results presented demonstrate that promoters for the N4 virion RNA polymerase can be found in heterologous DNA at a frequency of one per few thousand nucleotides. They are specific in that, where ever we have determined unambiguously the starting RNA nucleotide, only a single start site is used for initiation in the promoter region. However, all sites determined have low activity relative to the natural N4 promoters. Somewhat similar results have been reported for T7 RNA polymerase with some templates (22), though the initiation sites were not

Figure 7. Determination of the relative promoter strengths of pBR322 sites of transcription initiation by hybridization of [$\gamma^{32}\text{P}$]GTP initiated RNA to Southern blots. Conditions as in Materials and Methods. A: pBR322 template, B: pBR(K) template. 1: DNA-DNA hybridization with nick-translated self DNA, 2: RNA-DNA hybridization.

determined. Recently, a search for the minimal *lac* operator was made using a procedure very similar to the above (23). The approach is useful in cases where *in vivo* recognition sites are few in number.

The studies presented in this paper were all performed using denatured DNA, which was the template used to identify the natural promoters of the virion RNA polymerase (7). Since N4 has a linear duplex genome, this may seem an unnatural template. Indeed, many polymerases utilize single-stranded templates at elevated enzyme concentrations with reduced specificity (24). However, the natural N4 promoters are used with *in vivo* specificity when they are present on denatured or single-stranded template DNA (5,8). The N4 virion RNA polymerase shows absolutely no activity on duplex DNA, unless the template is supercoiled to physiological levels and *E. coli* single-stranded DNA-binding protein (SSB) is present (5). Some of the weak promoters, like pBR322 *Hinf*I 506-1, are active on supercoiled SSB-activated template (P. Markiewicz, unpublished). Therefore, the above results are likely to be relevant to the actual promoter structures recognized on duplex template by the virion polymerase.

Figure 9. Sequences of weak sites of transcription initiation for the N4 virion RNA polymerase on heterologous templates aligned for maximal similarity to N4 promoters.

where NN and N'N' stand for any nucleotides that are complementary (N complementary to N'). The sequences are aligned with respect to +1 and the initiation site(s) is marked by an arrow. When more than one arrow is present, it indicates that the 5' end of the RNA product has not been unambiguously determined by sequencing of the 5' end of the transcript. Wild type promoter conserved sequences have been underlined and inverted repeats have been indicated by arrows.

Sequences are listed in order of decreasing strength relative to the N4 P1 promoter. Taking into account the presence of conserved features and promoter strength, these sequences can be divided into two groups. Those near the top of the figure can be considered derivatives of a wild type N4 promoter where substitutions, and/or insertions, and/or deletions have occurred. Their representation in Figure 9 reflects such interpretation. In all cases, the extent of sequence similarity revealed by this representation is surprising. Such degree of similarity is reflected in the promoter strength of this group of sequences which differ as much as 20-300 fold with the bottom group of sequences presented in Figure 8.

Inspection of these stronger variants suggests that both the conserved sequences and the inverted repeats are important for activity. However, definite conclusions can not be drawn since, unfortunately, the sequences

identified differ by more than one base change from the natural promoters. N4K* and ØX174 RsaI 472 have similar strengths; the latter, although closer to the wild type consensus sequence, is missing the inverted repeats while N4K* differs more from the consensus but contains inverted repeats. We speculate that the inverted repeats are important for activity although its relevance can not be directly assessed here. Results of recent experiments using oligonucleotide site-directed mutagenesis, where the non-conserved central bases at the inverted repeats have been changed, indicate that the presence of the inverted repeats contributes considerably to promoter strength (26, A. Glucksmann, unpublished). The two promoters contained in the pBR322 Hinf 1 fragment are the strongest non-N4 promoters identified up to date. They share considerable sequence similarity to the wild type promoters except for localized regions where there are "inserted bases". We believe that the description of both promoters in Figure 9 is warranted since N4 virion RNA polymerase recognizes its promoters in single-stranded template which would permit the flexibility required for looping out sequences. Variability is already present at the wild type P3 promoter where a base is absent at position -3. Preliminary results indicate that the presence or absence of a base at the position does not alter promoter strength (A. Glucksmann, unpublished) suggesting that, at least, small variations in the distance between two conserved sets of sequences are allowed. Further experiments are required to test the limit of flexibility.

Little can be concluded from an analysis of the last six sequences presented in Figure 8 since they constitute extremely weak promoters. They all contain, however, a minimum of two elements and, in general the CTTX_nTGG sequence. Whether this combination is sufficient for unidirectional and specific initiation remains to be determined.

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