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The early promoter of bacteriophage Mu: definition of the site of transcript initiation

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ABSTRACT

The early promoter of bacteriophage Mu has been identified and characterized by a nitrocellulose filter binding assay and analysis of RNA products transcribed *in vitro* and *in vivo*. A tight, heparin resistant, RNA polymerase-DNA binding site overlaps the left Mu Hind III site. *In vitro* [γ^{32}P] ATP initiated transcription begins approximately 25 nucleotides to the right of the Hind III restriction site. Dinucleotide initiation with ApA and S1 mapping of *in vitro* and *in vivo* transcripts shows that transcription is initiated 1028 base pairs from the Mu genetic left end.

INTRODUCTION

Bacteriophage Mu is the most active transposable element known. During the replicative cycle, the virus transposes to 50 different sites in the bacterial genome within an hour (1,2,3). Mu transposition can be carried out *in vitro* (4,5) so it provides an important system to dissect the process of replicative transposition. However, Mu is also a temperate virus capable of stable integration into the host chromosome. Intracellular regulation of Mu transposition reflects an important choice between replication and lysogeny.

The genetic switch and the genes which determine whether lysis or lysogeny will be the selected mode of infection are located at the left or immunity region of the Mu map (6). Lysogeny and immunity to further viral infection of the host are maintained by the product of the repressor gene (*cI*) (6,9). Plasmids containing the left Mu Hind III fragment confer immunity to super infection with Mu (8), and *in vitro* transcription studies indicate that the repressor transcript is made right to left (10).

Upon inactivation of temperature sensitive repressor mutants by thermoinduction, transcription of the early region begins (11). Early messenger RNA is synthesized rightward across Mu DNA. The length of the transcript is unknown although the data of van Meeteren and van de Putte suggests *rho* independent termination takes place in or before the *C* gene (14). Transcription begins immediately following infection or induction of

a temperature sensitive lysogen and reaches a peak after four minutes (11-14). In the next four to five minutes, the amount of early transcript decreases due to production of the ner protein which acts as a self-regulating repressor (10,14,15).

R-loop analysis indicates that the early transcript is probably longer than 6 kb. (J. Engler, personal communication) Proteins encoded by the early transcript include the products of the A and B genes which are required for Mu transposition and DNA synthesis (12,15,16). A number of small proteins which do not appear to be essential for transposition, are also presumed to be encoded in this region (6,17,18). Priess et al. (19) sequenced the left-most 1590 base pairs of Mu DNA and used this information to suggest locations for the promoters of cI (Pi) and the early transcript (Pe). They also showed by electron microscopy that RNA polymerase binds to this area.

In order to define the DNA sequence involved in regulating transposition, we have defined the starting point for mRNA synthesis in the early region of Mu. The Pe promoter straddles the left Hind III restriction site and initiates transcription of RNA rightward on the heavy DNA strand.

MATERIALS AND METHODS

Enzymes

Restriction enzymes Hinf I, Hind III, Hae III and Bal I, as well as bacterial alkaline phosphatase, were obtained from Bethesda Research Laboratories. E. coli DNA-dependent RNA polymerase was a gift from A-Young Woody and gave identical results to the enzyme prepared by Boehringer Mannheim. S1 nuclease was purchased from Boehringer Mannheim and polynucleotide kinase was a gift from Alan Morrison.

Radioactive Nucleotides

Preparation of $[\alpha$ & $\gamma^{32}P]$ nucleotides were done with HCl-free $[^{32}P]$ inorganic phosphate obtained from New England Nuclear. $[\alpha^{32}P]$ ATP was prepared by the method of Johnson et al. (20) and had a specific activity of 1000 Ci/mMole. $[\gamma^{32}P]$ ATP and GTP were synthesized as described by Maxam and Gilbert (21) also with specific activities of approximately 1000 Ci/mMole.

Other Materials

Cold nucleotide triphosphates, bovine serum albumin, dithiothreitol and brewers yeast tRNA were obtained from Sigma Chemical Corporation. Electrophoresis purity acrylamide and N, N' methylene-bisacrylamide were purchased from BioRad.

Preparation and Isolation of DNA

All DNA restriction fragments were prepared from the pBR322 derivative plasmid pPH03 (5) which contains 1600 base pairs of the Mu left end. Restriction digestions were carried out a minimum of two hours and fragments were resolved on 8% polyacrylamide, 0.3% bisacrylamide gels. EtBr stained bands were cut out, and DNA was eluted using an ECU-40 electroeluter from CBS Scientific. Resuspended DNA was made 0.3 M in sodium acetate, ethanol precipitated, washed, and resuspended in the appropriate buffer.

RNA Polymerase Mediated Binding of DNA to Nitrocellulose

Conditions for RNA polymerase binding to DNA and filtration through nitrocellulose were essentially those of Taylor and Burgess (22). Reactions contained 1.2 µgms of Hinf I restricted pPH03, resuspended in 25 µls of RNA polymerase binding buffer (PBB): 40 mM Tris-HCl, pH 7.9, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 µg/ml bovine serum albumin, and 10% v/v glycerol. 10 units of Hind III were added where indicated and incubation was carried out for 1 hour at 37°. Samples were incubated with 50 nM RNA polymerase for 10 minutes at 37° to allow binding to the DNA. The removal of restriction enzyme by successive extractions with phenol, chloroform, and ether prior to incubation with RNA polymerase gave identical results (data not shown). Heparin was then added to a final concentration of 100 µg/ml and incubation continued at 37° for 5 minutes.

The reactions were diluted with 250 µls PBB containing 100 µg/ml heparin and immediately filtered. Nitrocellulose paper obtained from Schleicher and Schuell was presoaked at least one hour in PBB without bovine serum albumin (PFB). The nitrocellulose strip was mounted on a hybridot box purchased from Bethesda Research Laboratories. Samples were filtered at the rate of 1 ml/minute and washed twice with 250 µls of PFB containing 100 µg/ml heparin. DNA was eluted from the filters by punching out the dots into eppendorf tubes and adding 25 µls of : 5 mM Tris/HCl pH8.0, 1% SDS, 4% Ficol 400, 0.005% bromphenol blue. After incubation for 30 minutes at 65° the samples were centrifuged, nitrocellulose dots were removed, and the samples were loaded onto an 8% acrylamide, 0.3% bisacrylamide gel and run at 150 volts until the dye ran off the gel.

IN VITRO Transcription of DNA

In vitro transcriptions were carried out in PBB containing 20 µg/ml tRNA in 25 µl reaction mixtures. Template DNA, RNA polymerase, and $[\alpha^{32}P]$ATP or $[\gamma^{32}P]$ATP were added to final concentrations of 10 nM, 50 nM and 5µM respectively. Incubation was carried out for 10 minutes at 37° to allow RNA

polymerase binding to template. Transcription was initiated by addition of the three remaining nucleotide triphosphates to 50 μM final concentration and 100 μg/ml Heparin. After 10 minutes at 37°, reactions were stopped by addition of 225 μls ice cold 0.3M sodium acetate and ethanol precipitation. Pellets were resuspended in 15 μl:80% formamide, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue, and run on an 8% acrylamide, 0.3% bisacrylamide, 50% urea sequencing gel for 150 min.

Dinucleotide initiation of *in vitro* transcripts is described by Moran *et al.* (23). Reaction buffer, DNA, and RNA polymerase concentrations used were the same as above. The specified dinucleotide was added to 150 μM concentration and followed by a 5 minute incubation at 37°. Transcription was initiated by addition of 2 μM CTP, GTP and UTP with $[\alpha^{32}P]$ATP added at 0.5 μM. After 1 minute, the reaction was made 150 μg/ml in heparin. Polymerization was allowed to proceed for 10 minutes and was then chased with 1.5 mM ATP, and 0.25 mM each of CTP, GTP, and UTP.

S1 mapping of IN VITRO and IN VIVO early transcripts

RNA was extracted from 25 ml cultures of *Escherichia coli* K 12 strains HB 101 and HB 101::Mu *cts* by the method of Bovre and Szybalski (24). Cells were grown to an A_{650} of 0.35 at a temperature of 32° and then induced at 43°. RNA was extracted four minutes after induction, ethanol precipitated, and divided into 10 fractions for hybridization and S1 mapping. RNA was also synthesized *in vitro* as described earlier, with cold ATP, from a Hinf I-Hae III DNA restriction fragment as template for S-1 mapping.

The DNA hybridization probe was a Hind III-Hae III restriction fragment, 5' end-labeled at the Hae III site using the blunt end labeling technique described by Maxam and Gilbert (21). Hybridization and S1 nuclease digestion were carried out as described by Berk and Sharp (25). Hybridization was carried out at 60° for 4 hours. Following S1 digestion, the DNA-RNA hybrid was ethanol precipitated, and resuspended in 15 μl: 80% deionized formamide, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue, and boiled 2 minutes before loading on a sequencing gel beside a Maxam-Gilbert sequence of the same DNA restriction fragment.

RESULTS

A search for an RNA polymerase binding site in the early region of Mu was conducted through nitrocellulose filtration. Duplex DNA normally passes through nitrocellulose filters whereas DNA in a complex with many proteins, including RNA polymerase, has been shown to be selectively retained on the

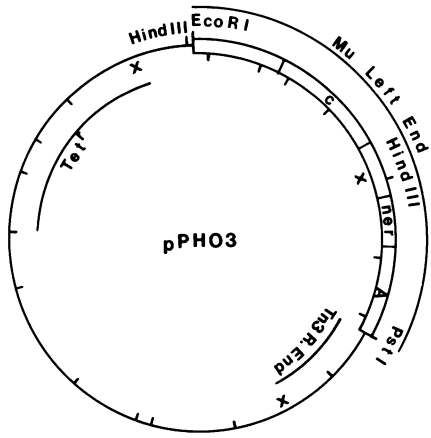

Fig. 1 - Physical map of plasmid pPH03. This 5400 base pair plasmid was derived from pKN61 (29) and contains the left end of Mu cloned between Pst I and EcoRl restriction sites in pBR322. The c, ner, and a fragment of the A gene of Mu are shown as are sites for Hind III, EcoRl, and Pst I restriction enzymes. Cleavage sites for Hinf I are indicated by lines on the inside of the circle and the fragments containing a strong RNA polymerase binding site are marked with an X.

filter (26-28). Heparin was added to remove RNA polymerase which was not tightly bound to DNA in open promoter complexes.

Plasmid pPH03 contains the left end of Mu cloned between Eco Rl and Pst 1 sites in pBR322 (Fig. 1). The left Mu genetic end is near the Eco Rl site and the Pst 1 site in the Mu A gene. In the Hinf I digestion of pPH03, three restriction fragments were retained on the filter following incubation with RNA polymerase (Fig. 2). Of these, the 700 and 516 bp fragments contain the tetracycline promoter and part of the Tn 3 right end respectively, derived from the original pBR322 plasmid (indicated by X's in Fig. I). The 712 bp Hinf I fragment, which also bound to the filter, contains the region which begins 650 bps from the left end of Mu and ends 32 base pairs inside the A gene. A more precise location for an RNA polymerase binding site in the Mu 712 bp fragment was demonstrated by further cleavage of the plasmid with Hind III. The plasmid contains two Hind III sites, one in the tetracycline promoter, and the other approximately 1000 bps from the Mu left end. When the plasmid is cut with Hind III, four new bands are generated from the 712 and 700 bp fragments (Fig. 2, lane d). The Mu 712 band generates fragments of 351 and 360 bps in length. The 700 band generates fragments of 602 and 98 bps. Of the new bands created by Hind III digestion, only the 351 bp Mu fragment is weakly retained on the

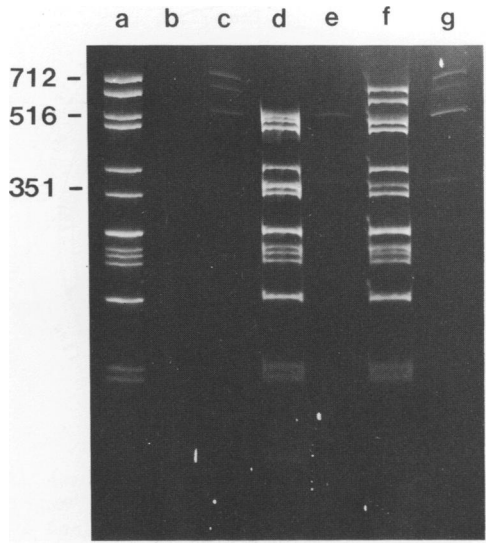

Fig. 2 - E. coli RNA polymerase complexes with plasmid DNA. Hinf I restriction fragments of plasmid pPH03 displayed in a 1% agarose gel were: a) given no treatment; b) adsorbed to nitrocellulose; c) incubated with RNA polymerase and then adsorbed to nitrocellulose; d) cleaved with Hind III restriction endonuclease; e) cleaved with Hind III, then incubated with RNA polymerase and adsorbed to nitrocellulose; f) cleaved with Hind III after pre-incubation with RNA polymerase; g) cleaved with Hind III after pre-incubation with RNA polymerase and then adsorbed to nitrocellulose.

filter. This region probably carries the leftward promoter for the repressor gene (7,19). The binding of the Tn3-516 bp fragment suggests the presence of a promoter sequence although none have clearly been shown. A possible promoter in the Tn3 right end, directed in to the element, has been observed (M. J. Casadaban personal communication).

Since the Mu promoter seemed to include the Hind III restriction site, an alternative approach was to see if RNA polymerase would protect the DNA from Hind III cleavage. This result had been suggested by unpublished results of Allet (See 19). Lane f of Fig. 2 shows that when preincubated with RNA polymerase, the majority of both the Mu 712 and Tet gene containing 700 bp fragments remain intact after addition of Hind III, and are bound to RNA polymerase when passed through nitrocellulose (lane g). Thus, RNA polymerase protects the DNA from Hind III cleavage.

An in vitro transcriptional assay was then used to show that RNA polymerase, demonstrated to be bound at the Hind III site, was functional in

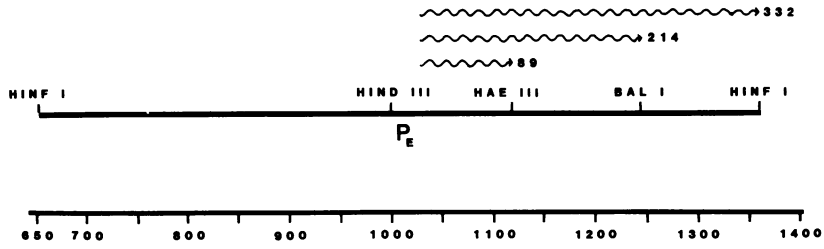

Fig. 3 - Map of the Hinf I fragment containing sequences from nucleotide 650 to 1360 of the Mu left end. Restriction sites used to generate templates for run-off transcripts are indicated. Represented above are the sizes of transcripts expected to be made from different templates.

the production of an RNA transcript. The Mu Hinf I fragment containing the Hind III site was purified as the template for transcription (Fig. 3). Further cleavages were made with Bal I, Hae III, or Hind III, as indicated, to change the sizes of the "run-off" transcripts. Figure 4 shows the $[\alpha^{32}P]$ATP labeled

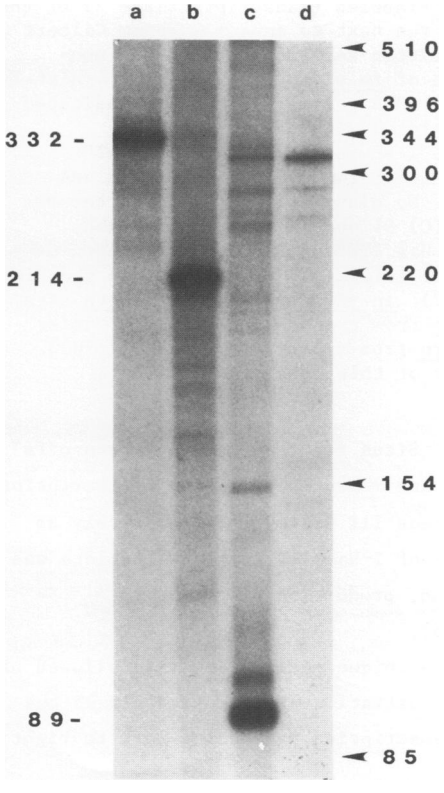

Fig. 4 - In vitro transcription of restriction fragments containing the Mu early promoter (see Fig. 3). Transcription was carried out with a) the Hinf I fragment; b) the Hinf I - Bal I fragment; c) the Hinf I - Hae III fragment; or d) the Hinf I - Hae III fragment cut with Hind III before addition of RNA polymerase. The positions of DNA size markers are indicated to the right, and the sizes of the major transcripts are indicated on the left.

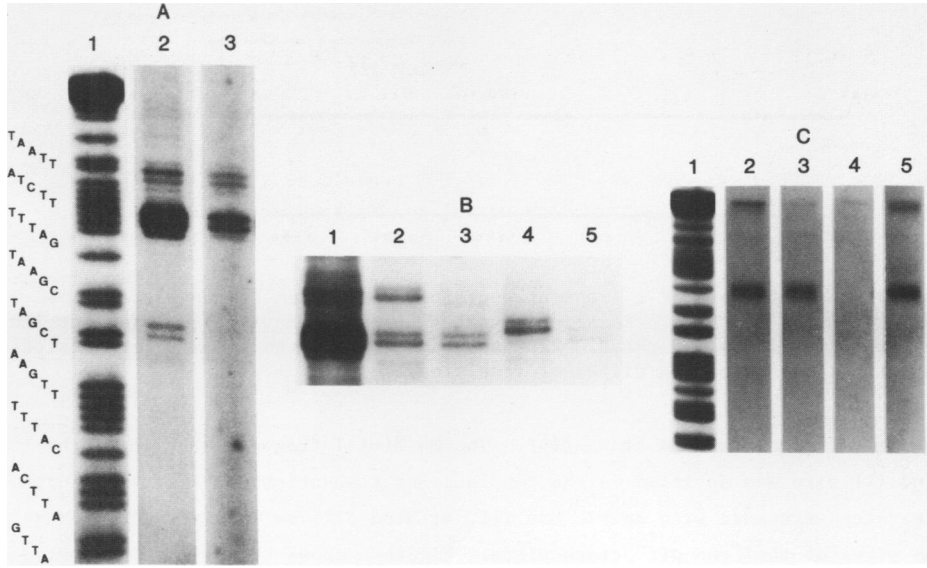

Fig. 5 - Mapping the early promoter initiation site. (A) In vitro [α^{32}P ATP]-labeled transcripts (lane 2), or [γ^{32}P ATP]-labeled transcripts (lane 3) of the Hinf I - Hae III restriction fragment were run next to an A + G Maxam Gilbert sequence of the fragment in lane 1. The written sequence is from the non-coding strand. (B) Dinucleotide initiation of in vitro transcription. Hinf I - Hae III template DNA was preincubated with RNA polymerase and the indicated dinucleotides before transcription was initiated with the addition of nucleotide triphosphates and heparin. Lane 1 shows the normal [α^{32}P]ATP labeled run-off. Run-off transcripts were initiated with dinucleotide ApA (lane 2), ApG (lane 3), and UpA (lane 4). No dinucleotide was added before addition of NTP's and heparin to lane 5. (C) S1 nuclease mapping of RNA hybridized to [^{32}P] end-labeled Hind III - Hae III* probe DNA. The S1 digestion products are shown for transcripts initiated: in vitro from the Hinf I - Hae III Mu restriction fragment (lane 2); in vivo from HB101::Mu cts after thermoinduction for 4 min (lane 3); in vivo from the non-lysogen HB101 after shifting to 42° for 4 min (lane 4); in vitro from supercoiled plasmid pPH03. Lane 1 contains the A + G sequencing ladder of this fragment.

transcripts produced from these templates. Sizes for the different "run-offs" were calculated at approximately 330, 215, and 90 nucleotides for transcription using the Hinf I, Hinf I-Bal I, and Hinf I-Hae III fragments respectively as template (Fig. 4, lanes a,b,c). When the Hinf I-Hae III fragment template was treated with Hind III prior to transcription, production of the 90 bp transcript was disrupted completely (lane d).

The termination of each transcript at a unique restriction site allowed us to determine the common site of transcript initiation at approximately 25 bps to the right of the Hind III site, with transcription proceeding left to right (illustrated in Fig. 3).

Fig. 6 - The Mu early promoter. The sequence of the early promoter is given, with the pribnow box and -35 binding regions underlined. Transcription proceeds rightward and is initiated with the indicated nucleotide.

A more precise localization of the initiating nucleotide was attempted by running the Hinf I - Hae III transcript on a sequencing gel beside a Maxam - Gilbert sequence of the Hind III - Hae III DNA fragment ^{32}P-labeled at the Hae III end. Fig. 5A lane 2 shows the labeled transcript as a set of bands; a relatively dark doublet with a lighter band below. Labeling of the transcript with γ^{32}P ATP (lane 3) shows the same pattern indicating the transcript is either initiating rather ambiguously at A's in the DNA sequence, or is being initiated at a unique adenosine residue and is terminated at the 3' end in an unusual manner, creating two transcripts with identical 5' ends but differing in length by one or two nucleotides at the 3' end.

This technique still fails to demonstrate the exact length of the transcript due to the fact that, on a denaturing gel, RNA runs slower than single or double stranded DNA of identical length and sequence. Initiation of transcription at one adenine residue was confirmed by dinucleotide initiation. By incubating the template with RNA polymerase and the dinucleotide (NpN) corresponding to the first and second nucleotides of the transcript, one can selectively initiate transcription only when the proper dinucleotide is used. Figure 5 part B shows the products of transcription when different dinucleotides are used to initiate transcription from a Hinf I - Hae III template. These transcripts differ from the ordinary transcripts in that they do not possess a triphosphate at the 5'end. This alters their apparent size on our sequence gel by an approximate 2-3 nucleotides. In all cases, some normal transcripts were initiated even following heparin treatment, as is demonstrated in the control where no dinucleotide was added to the initiation complex.

A strong doublet was generated using the dinucleotide ApA to initiate transcription and appeared, as expected, running approximately 3 nucleotides above the normal transcript. A very faint dinucleotide appeared one position lower using ApG, and using UpA, a fairly strong doublet appears another three positions further down, as well as a faint doublet showing up one position above the ApA initiated transcript. These results agree with the sequence shown in Figure 6. The UpA generated run-off was not consistently observed in

other trials.

The first nucleotide of the early transcript then, is the adenosine residue 27 bp to the right of the Hind III site and is designated in Figure 6. Even when a dinucleotide, which possesses no phosphates on the 5'end, is used to initiate transcription, two transcripts differing in size by one nucleotide are always produced no matter which dinucleotide is used. This indicates the difference between the two bands or transcripts in each doublet must occur at the 3' end. Run-offs from other templates produced only single bands.

Finally, we wished to show that the early message produced in infected cells was also initiated from the same promoter and with the same nucleotide. To do this, we isolated *in vitro*, or *in vivo* RNA, hybridized it under RNA-DNA hybrid forming conditions to a Hind III - Hae III end-labeled DNA probe, and followed with S1 digestion to remove the single strand tails. Figure 5, Part C shows the S1 ss DNA products run beside an A+G ladder of the same DNA fragment which had been used as the probe. Both the *in vitro* and *in vivo* transcripts generated S1 fragments of identical size (lanes 2 and 3). These line up opposite the adenine 27 nucleotides to the right of the Hind III site as indicated by the DNA sequence of the strand complementary to the transcript. Some ambiguity arises as the result of a tendency for S1 nuclease to hesitate one nucleotide from the blunt end of the RNA-DNA hybrid, and thus we see two or three bands representing the transcript.

The absence of full length DNA probe in the *in vivo* S1 mapping, indicates that there is no significant production of early transcript initiated from an upstream promoter; at least not before the four minute sampling time following induction when the RNA was isolated.

In the last part of this experiment, we added the supercoiled plasmid, pPH03, as our template in the *in vitro* production of RNA. This was an attempt to determine whether or not transcription from the early promoter might be affected by integration of linear Mu DNA into the supercoiled host chromosome. Figure 5C lane 5 shows the S1 product of the hybridization of transcript from pPH03 is not significantly different from early transcript made *in vivo* or *in vitro* from linear DNA.

DISCUSSION

Similar to λ phage in many respects, Mu is a temperate phage which can make one of two choices following infection of a host cell. In Mu, however, both lytic and lysogenic pathways require integration of Mu DNA into the host chromosome. To achieve integration, Mu must first produce the *A* protein and

for more efficient integration (10 X higher) the B protein and possibly other early functions (15,17). In λ, integration and DNA replication functions are coded for by genes on different regions of the viral DNA and are under the control of different operators which compete with one another. Mu must use a subset of the same proteins required for lytic reproduction, for lysogeny as well. For Mu to be a successful lysogen, it must transcribe the early region without initiating DNA replication and transposition, killing the host, or expressing the late genes. This requires some interesting form of regulation. The early message has been previously demonstrated to initiate immediately after infection or induction of a lysogen containing a thermosensitive repressor, and to terminate under lytic conditions in or before the C gene.

In this paper, we have shown that transcription in vitro, and during early development in vivo, is initiated from a promoter located over the left Hind III restriction site. Transcription is initiated with ATP 27 nucleotides to the right of the Hind III cleavage site, and proceeds rightward. Sequence analysis shows this is the only obvious promoter proximal to the ner and A genes, and hybridization of in vivo RNA produced by induced lysogens four minutes after induction, implies the absence of upstream promoters. If the switch which selects lysogeny over lysis is controlled at the level of transcript initiation, this would require immediate repression of the early promoter as Mu DNA enters the host. At this point, it is not clear whether decreasing the amount of early transcript, either by regulating the early promoter characterized in this paper, or by further induction of a weaker promoter, would be enough to direct the infecting virus into lysogeny. kil and C functions would have to be so low as to have no noticeable effect, while A and B protein levels would be sufficient to direct integration with no further transposition. An especially attractive hypothesis is that this operon contains additional regulatory features (i.e. transcription terminators or post transcriptional control). This might explain Mu behaviour in him A mutant strains (32).

Several lines of evidence indicate that the Pe promoter is autonomous and relatively efficient. Our studies show that RNA polymerase forms a stable heparin-resistant complex with Pe and initiates properly in vitro. In vivo studies demonstrate that the early transcript accounts for over 1% of the total mRNA synthesis in the first few minutes after induction (11, 13, M. Pato, personal communication). The strength and autonomy of the early promoter suggests the obvious advantage Mu has over other transposable elements. Many elements examined to date have weak promoters and very low rates of

transposition due to tight regulation of the transposase gene. Under lytic conditions where Pe is active, Mu has the highest transposition efficiency known; 50-100 events/hour.

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