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In vitro transcription of bacteriophage ϕ29 DNA. Correlation between *in vitro* and *in vivo* promoters

Rafael P.Mellado, Isabel Barthelemy and Margarita Salas

Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain

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ABSTRACT

The Ø29 DNA in vitro transcription initiation sites have been accurately mapped by S1 protection experiments. The results obtained indicated that the B. subtilis RNA polymerase containing the σ^{43} subunit basically recognized the same set of Ø29 promoters in vitro as those used in vivo. In addition, the sequence of the Ø29 early A2a promoter used both in vitro and in vivo has been determined as well as the precise nucleotide where initiation of transcription from the C2 promoter occurs in vitro.

INTRODUCTION

The B. subtilis phage Ø29 contains a linear double-stranded DNA 18,000 base pairs long (1) with the viral protein p3 covalently linked at the two 5' ends (2). The Ø29 late genes are clustered in the middle of the genome, flanked by the early genes, which are localized at both ends on the genetic map (3). The B. subtilis RNA polymerase holoenzyme binds in vitro to seven specific sites in the viral genome, A1, A2, A3, B1, B2, C1 and C2 as visualized by electron microscopy (1, see Fig. 1) and initiates transcription at these seven sites plus three other ones, two of them, A1III and B1I, are binding sites for the E. coli RNA polymerase and the other one does not correspond to any known binding site, as determined by R-loop analysis under the electron microscope (4,5). All these initiation sites correspond to Ø29 early transcription except the A3 one, where late transcription can initiate after prolonged incubation with the B. subtilis or E. coli RNA polymerase holoenzymes (4). The in vivo transcription initiation sites have been accurately located by S1 mapping experiments along the Ø29 genome, close to the bacterial RNA polymerase binding sites, and the promoter sequen-

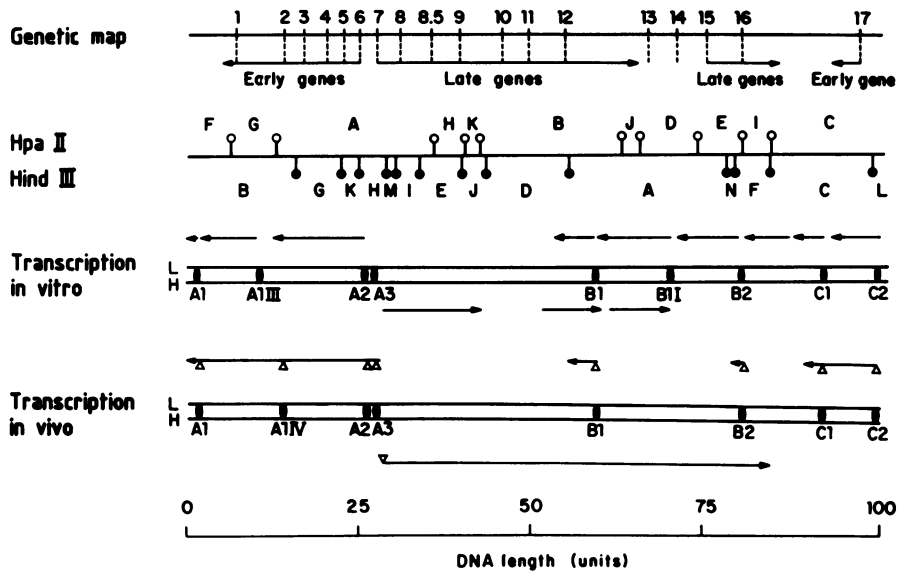

Figure 1. Ø29 genetic, physical and transcriptional maps. The genetic map is adapted from that of Mellado et al. (3). The black dots, A1, A2, A3, B1, B2, C1 and C2 are B. subtilis RNA polymerase binding sites and A1III, A1IV and B1I are E. coli RNA polymerase binding sites (1,4). Arrows indicate the direction and extent of transcription. Early transcription takes place from the light (L) strand of Ø29 DNA and late transcription occurs from the heavy (H) DNA strand in the opposite direction. In vivo transcription initiation sites are indicated by open triangles. In the in vivo transcription map, the arrows extend to the maximal length protected in the S1 mapping experiments (Barthelemy et al., submitted). ♀ HpaII sites; ♀ HindIII sites.

ces of eight of them have been determined (Barthelemy, Salas and Mellado, submitted; Mellado, Barthelemy and Salas, submitted).

To correlate the Ø29 DNA in vivo promoter sequences with those utilized in vitro by the B. subtilis RNA polymerase holoenzyme, the in vitro transcription initiation sites have been accurately mapped by S1 protection experiments. The results obtained confirmed that the B. subtilis RNA polymerase holoenzyme initiates transcription in vitro at the same positions used in vivo in the Ø29 genome. In addition, the precise location and the sequence of the early viral promoter A2a has been determined. The direct determination of the nucleotide where transcription initiation from the C2 promoter occurs in vitro shows a good correlation with the S1 mapping data.

MATERIALS AND METHODS

a) Bacterium and bacteriophage strains. Reagents and enzymes.

B. subtilis 110NA trp$^-$ spoA$^-$ su$^-$ was used as host for the bacteriophage Ø29 growth. Restriction endonucleases were from New England Biolabs, nuclease S1 and the RNases from PL Biochemicals, calf intestinal alkaline phosphatase and T4 polynucleotide kinase from Boehringer Mannheim, {γ-^{32}P}ATP (~3000 Ci/mmol) and {γ-^{32}P}GTP (10 Ci/mmol) were from Amersham International. The *B. subtilis* RNA polymerase holoenzyme was prepared as described (1).

b) Preparation and labelling of nucleic acids, S1 mapping and DNA sequence analysis.

Labelling of DNA fragments at their 5'-termini with polynucleotide kinase and {γ-^{32}P}ATP was as described (6). Purification of double-and single-stranded DNAs by diffusion from polyacrylamide gels and the conditions for the protection of 5'-end labelled DNA to the S1 digestion were as described (Barthelemy et al., submitted). DNA sequencing reactions were as described (7) with some modifications (8).

c) In vitro transcription. Preparation of RNA.

The incubation mixture for the *in vitro* transcription was as described (4), except that 100-200 ng of the indicated purified Ø29 DNA fragments were used as templates. The RNA was extracted with hot phenol (9) twice and recovered by two cycles of ethanol precipitation. The total amount of RNA synthesized in each reaction was used for the S1 mapping experiments. Radioactive labelling of the RNA at the 5' end was done in the same manner by incubation with 50-100 µCi of {γ-^{32}P}GTP. The labelled RNA was fractionated by electrophoresis on 4% polyacrylamide gels in the presence of 7 M urea, purified by diffusion out of the gel (6) and sequenced by partial digestion with a set of RNases (10). Preparation of the *in vivo* made RNA was as described (Barthelemy et al., submitted).

RESULTS AND DISCUSSION

a) Transcription initiation within the HindIII H fragment.

In vivo Ø29 early transcription initiates at two main promoters in the HindIII H fragment, around the A2 binding site,

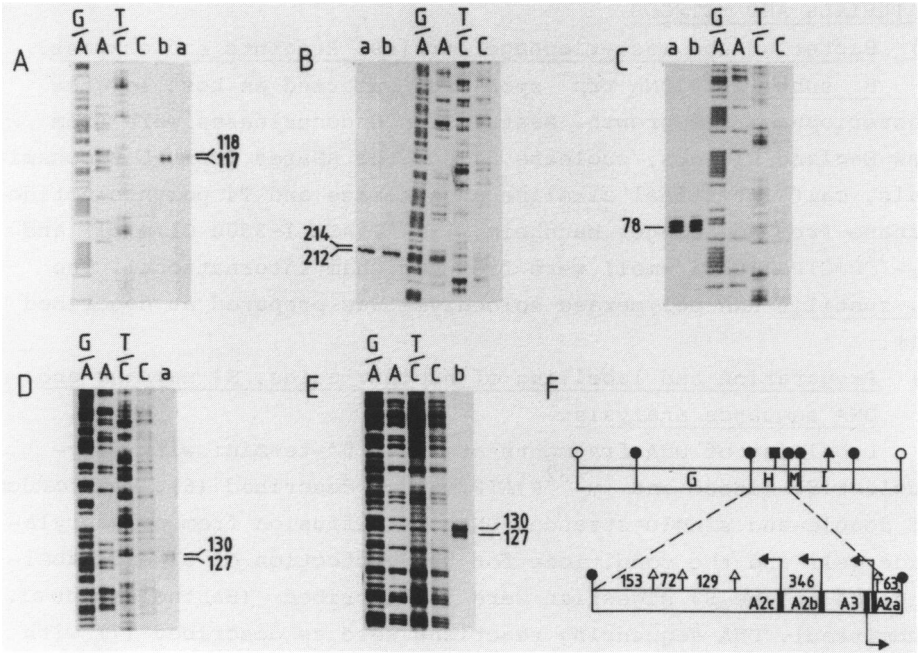

Figure 2. Transcription initiation within the HindIII H fragment, at the promoters A2c (A), A2b (B), A3 (C) and A2a (D,E). Sequencing reactions are from the late strand of the 346 base pairs MnlI subfragment from fragment HindIII H (A-D) or from the late strand of the 672 base pairs HindIII-HinfI subfragment from fragment HindIII B (E). a) oligonucleotides protected from S1 digestion by the in vivo made RNA; b) oligonucleotides protected from S1 digestion by the in vitro made RNA. F) Physical map of the region, HindIII, HpaII, BclI, AccI, MnlI. Arrows at the extension indicate the direction of transcription, numbers are the sizes of the MnlI subfragments in base bairs and vertical bars indicate the relative positions of the Ø29 promoters within the HindIII H fragment. Capital letters inside the map refer to the HindIII fragments.

namely A2b and A2c (Mellado et al., submitted; see Fig. 2F). To determine the in vitro initiation sites, the HindIII H fragment was used as template in the presence of the B. subtilis RNA polymerase holoenzyme containing the σ^{43} subunit; the in vitro made RNA was hybridized to the 5'-end labelled early strand of the 346 base pairs MnlI subfragment from fragment HindIII H (Fig. 2F) and the length of the oligonucleotides protected from S1 digestion determined by fractionation on denaturing polyacrylamide gels, side by side with the nucleotides resulting from sequencing

reactions. As an internal control, the equivalent oligonucleotides protected by the *in vivo* made RNA were fractionated in parallel. Figure 2A and B shows that oligonucleotides 117-118 and 212-214 bases long were protected *in vivo* and *in vitro* by the transcripts initiating at the A2c and A2b promoters, respectively, sizes that coincide with the ones previously reported for the *in vivo* transcripts (Mellado et al., submitted). There was also coincidence between *in vivo* and *in vitro* initiation of transcription when a similar experiment was carried out for the A3 promoter, either when the HindIII H fragment itself was used as template *in vitro* in a run-off transcription assay and the 5'-end labelled late strand from the same fragment was used in the S1 mapping, where an oligonucleotide around 78 bases long was protected (Fig. 2C), or when the HpaII A fragment (Fig. 2F) was used as *in vitro* template (not shown).

Figure 2D shows the length of the 5'-end labelled oligonucleotide from the early strand of the 640 base pairs AccI-BclI subfragment from the HpaII A fragment (Fig. 2F),protected by hybridization to the *in vitro* made RNA using the HindIII H fragment as template. The transcription initiation point matches with the one used *in vivo* by the *B. subtilis* RNA polymerase at the so called A2a promoter, as determined in the equivalent S1 mapping experiment (Fig. 2E).

b) *Transcription initiation at the left end and the middle part of the Ø29 genome*.

Two weak promoters were reported to function *in vivo* at the Ø29 DNA left end, the A1 and A1IV promoters (Mellado et al., submitted), the latter located close to an *E. coli* RNA polymerase binding site for which the equivalent *in vitro* transcription initiation site could not be determined by R-loop analysis (4). According to this result, no protected oligonucleotide was detected when the RNA synthesized *in vitro* by the *B. subtilis* RNA polymerase, using the HindIII B fragment as template (Fig. 3C), was hybridized to the 5'-end labelled early strand of the 672 base pairs HinfI subfragment. As shown in Fig. 3A around 166 nucleotides from the 5'-end labelled early strand of the 260 base pairs HinfI subfragment were protected (Fig. 3A, lane b), a size coinciding with the one protected from the same sub-

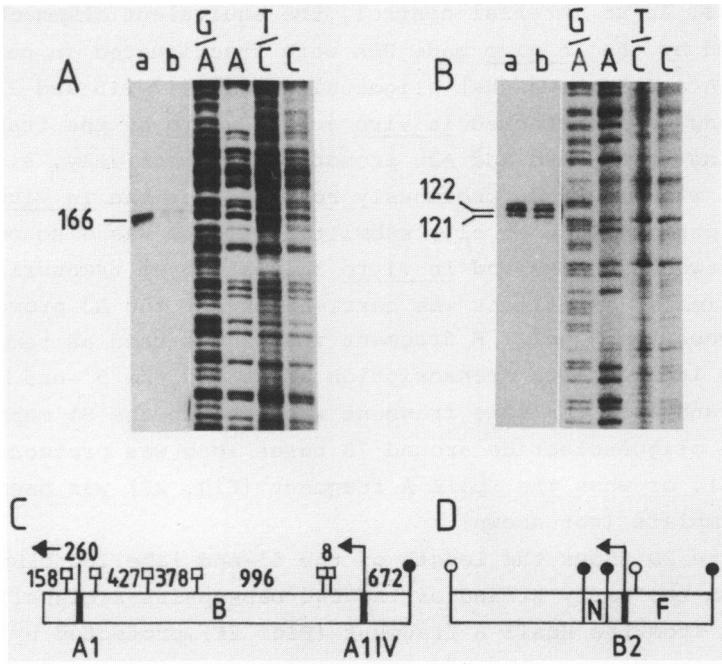

Figure 3. Transcription initiation at the A1 (A) and B2 (B) promoters. Sequencing reactions are from the late strand of the 672 base pairs HindIII-HinfI subfragment from fragment HindIII B (A) or from the late strand of the 880 base pairs HindIII-EcoRI subfragment from fragment HindIII D (B). a) oligonucleotides protected from S1 digestion by the in vivo made RNA; b) oligonucleotides protected from S1 digestion by the in vitro made RNA. C and D) Physical map of the A1 and B2 regions, respectively, HindIII, HpaII, HinfI. Arrows indicate the direction of transcription, vertical bars indicate the relative position of the Ø29 promoters and numbers are the sizes in base pairs of the HinfI subfragments from fragment HindIII B (C). Capital letters inside the maps refer to the HindIII fragments.

fragment by the in vivo early transcript initiating at the A1 promoter (Mellado et al., submitted; Fig. 3A, lane a). From these results it can be concluded that the A1 promoter, unlike the A1IV one, seems to be recognized in vitro by the B. subtilis RNA polymerase.

Two other weak early promoters work in vivo at the middle part of the Ø29 genome, the B1 and B2 promoters, for which a regulatory function in modulating the Ø29 late expression has been suggested (Barthelemy et al., submitted). When the in

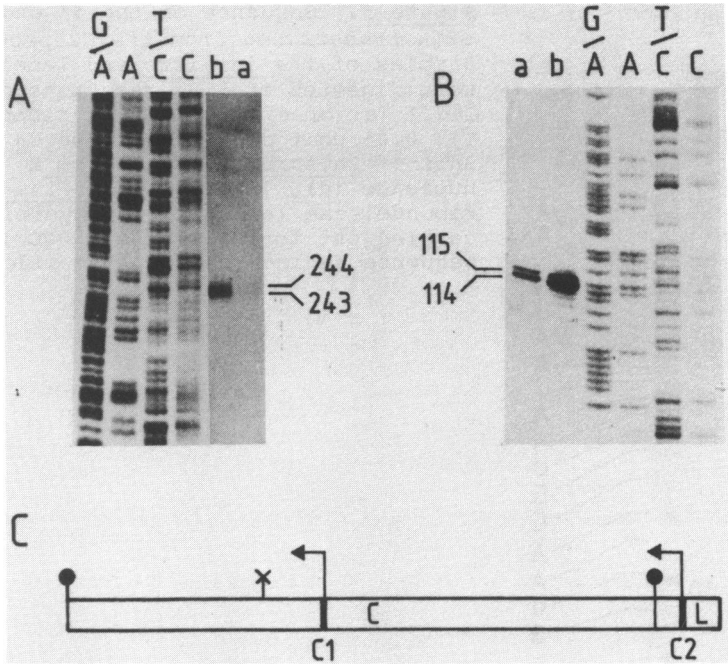

Figure 4. Transcription initiation at the C1 (A) and C2 (B) promoters. Sequencing reactions are from the late strand of the 346 base pairs MnlI subfragment from fragment HindIII H. a) oligonucleotides protected from S1 digestion by the in vivo made RNA; b) oligonucleotides protected from S1 digestion by the in vitro made RNA. C) Physical map of the region. HindIII, EcoRI. Arrows indicate the direction of transcription and vertical bars indicate the relative position of the Ø29 promoters. Capital letters inside the map refer to the HindIII fragments.

vitro made RNA using the HpaII E fragment as template was hybridized to the 5'-end labelled early strand of the HindIII F fragment (Fig. 3D), a nucleotide of 121-122 bases was protected from the S1 digestion (Fig. 3B, lane b), exactly of the same size as the equivalent one protected from the same DNA fragment by in vivo early RNA synthesized from the B2 promoter (Mellado et al., submitted; Fig. 3B, lane a). Analogously, an oligonucleotide of 740 bases was protected from the 5'-end labelled early strand of the 1100 base pairs HindIII-HpaII subfragment from the HpaII B fragment (see Fig. 1) by either RNA made in vivo or in vitro, confirming that the in vitro initiation of transcription occurs at or close to the B1 promoter used in vivo (results not shown).

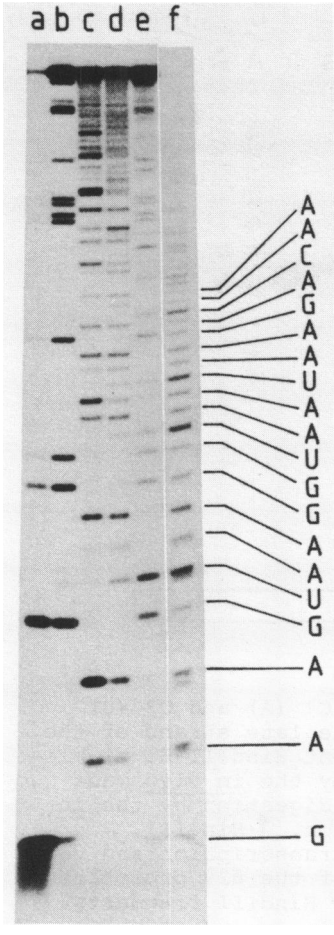

Figure 5. Sequence of the 5' end of the mRNA transcribed from the C2 promoter. Samples of the in vitro end-labelled mRNA were digested in 5 µl reactions containing 1 (a) or 0.1 (b) unit of ribonuclease T1; 0.05 unit of ribonuclease U_2 (c), 1 unit of Physarum polycephalum M ribonuclease (d), 1 unit of Bacillus cereus ribonuclease (e). Alkaline hydrolysis was carried out for 15 min. at 90ºC (f). The sequence is indicated on the side.

c) Transcription initiation at the right end of the Ø29 genome.

The in vitro made RNA using the EcoRI C fragment as template (Fig. 4C) protected from S1 digestion an oligonucleotide 114-115 bases long from the 5' end labelled early strand of the HindIII L fragment (Fig. 4B, lane b), showing again the coincidence with the in vivo initiation of transcription at the main early promoter C2 (Mellado et al., submitted; Fig. 4B, lane a). As for the other in vivo reported early promoter at the right end of the Ø29 DNA molecule, the C1 promoter, again a coincidence in the initiation of transcription in vivo and in vitro was found. The in vitro synthesized RNA using the HindIII C fragment as template (Fig.

4C) protected from S1 digestion an oligonucleotide of 243-244 bases from the 5'-end labelled early strand of the EcoRI fragment, which coincides with the in vivo result (Fig. 4A) and is in good agreement with the initiation reported in vitro (11). To check further the S1 mapping results, the Ø29 HindIII L fragment containing the C2 promoter was used as template to label in vitro the 114-115 bases long transcript at its 5' end with {γ-^{32}P}GTP since, as shown by Davison et al. (12), this transcript initiates with GTP. The sequence of the labelled RNA (Fig. 5) showed that the initiation of transcription really takes place 1-2 nucleotides downstream the position mapped by the S1 protection experiments, confirming the general validity of the S1 mapping results.

From all the results presented, it can be concluded that the B. subtilis RNA polymerase holoenzyme recognizes the same promoters in vivo and in vitro with the exception of the A1V promoter which is an in vivo weak one (Barthelemy et al., submitted) and was not detected in vitro before (4).

Figure 6 shows the DNA sequence for all the Ø29 in vitro promoters recognized by the B. subtilis RNA polymerase containing the σ^{43} subunit. All of them coincide with the ones previously shown to function in vivo (Mellado et al., submitted) being the sequence for the A2a promoter a new one reported in this paper. Comparison of these sequences with those of the previously inferred ones as Ø29 in vitro promoters allows to correlate the G3b, G3a, A1 and G2 promoters (13) with the ones named here A2c, A2b, A1 and C2, respectively.

The Ø29 late transcription is controlled by the product of the viral gene 4, protein p4 (1), being the A3 promoter the main Ø29 late one, which is recognized in vitro by the RNA polymerase with the σ^{43} subunit, probably due to the homology shared at the -10 region by the A3 promoter with that of the Ø29 early ones (Fig. 6). Interestingly enough, the A2a promoter carries the sequence TATGTTTCA which matches in eight out of the nine bases with the sequence TATGTATCA carried by the A3 promoter in a similar position. In addition, both promoters share the sequence TAGTACTA at the transcription initiation point and the A2a promoter was shown to work better in vivo when the viral infection was

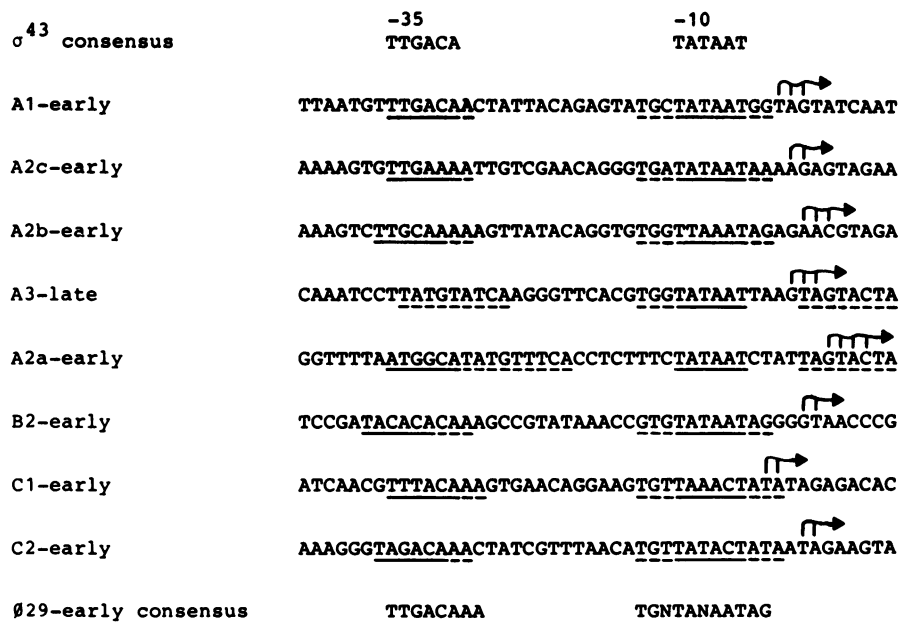

Figure 6. Comparison of the Ø29 early and late promoter sequences. Wavy lines indicate the initiation sites and the direction of transcription. Solid lines underline sequences in the Ø29 promoters which correspond to the consensus ones recognized by the B. subtilis RNA polymerase with the σ^{43} subunit. Broken lines underline regions of homology among the different promoters. The -35 and -10 early Ø29 consensus sequences have been derived (Mellado et al., submitted).

carried out in the absence of chloramphenicol (Barthelemy et al., submitted). This might suggest the existence of a temporal control of the Ø29 gene transcription in vivo which ensures the expression, not only of late genes, but also of those early ones needed at late times after infection. Thus, the transcription, among other early genes, of gene 4, whose product controls the viral late transcription, could be specifically directed at late times from the A2a promoter whereas the gene could be expressed from the main early promoters A2b and/or A2c at early times after infection. In vitro transcription studies on the function of protein p4 in the modulation of the expression of Ø29 promoters currently in progress, should help in the understanding of the control of Ø29 gene transcription.

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