Volume 16 Number 10 1988 Nucleic Acids Research

Bacteriophage T7 late promoters with point mutations: quantitative footprinting and *in vivo* expression

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Received January 12, 1988; Revised and Accepted April 14, 1988

INTRODUCTION

The viral encoded RNA polymerase of bacteriophage T7 shows a high degree of template specificity because it initiates transcription only at the late promoters (1). The late promoters of T7 are considerably different from bacterial promoters in that they consist of a highly conserved uninterrupted sequence that is 23 base pairs long (2). We have previously described a set of 18 cloned T7 late promoters that contain point mutations (3). These point mutations can be divided into two types, called A and B, on the basis of the in vitro transcription activity of the promoter and the location of the mutation within the promoter. We have used quantitative footprinting techniques to determine the binding constants for the interaction of T7 RNA polymerase with the mutated late promoters (4). Our results support the hypothesis that T7 late promoters consist of two functional domains. The conserved base pairs upstream from -6 are primarily involved in the binding of the polymerase in a closed complex, whereas the base pairs downstream from -5 are primarily involved in the initiation of transcription.

The pKCT7P series of plasmids contain the E. coli galK gene under the transcriptional control of either the standard or a mutated T7 late promoter (3). This enabled us to determine the activity of the promoters in vivo by measuring the specific activity of galactokinase. The effects of the various point mutations on promoter activity in vivo are consistent with observations made in vitro (3).

Because T7 late promoters contain a Hinf I site (5), it has been possible to make mutated promoters with deletions of the base pairs upstream from -10. In these mutated promoters, the region from -11 to -18 is replaced by a new segment of DNA, resulting essentially in promoters with multiple substitutions. Four promoters of this sort have been constructed, and all are active in vitro (6,7). Since these four promoters have different

sequences in the -11 to -18 region, it seems that the conserved base pairs upstream from the Hinf I site are not be essential for promoter function. However, all of these promoters have some base pairs in the -11 to -18 region that are identical to those found in the consensus sequence of T7 late promoters, leaving open the possibility that base pairs in this region interact specifically with the polymerase but that they need not all be present to have some promoter activity. One of these partially active promoters contains 5 base pairs from -15 to -19 which are identical to the consensus sequence of wildtype promoters (7). When these base pairs are replaced by DNA with no homology to the consensus sequence, the in vitro transcription activity is abolished. This indicates that there are sequence specific interactions between the polymerase and the -15 to -19 region.

MATERIALS AND METHODS

Promoters With Point Mutations

The making of the T7 late promoters with point mutations has been described previously (3). The promoter in pKCT7P was used as the standard for these assays. This promoter is not a naturally occurring T7 late promoter, but a hybrid consisting of the left half of a class II promoter and the right half of a class III promoter (3). The pKCT7P promoter is actively utilized by T7 RNA polymerase both in vitro and in vivo, and it has the consensus sequence of T7 late promoters from -17 to +6. It can be isolated on a 37 bp Eco RI - Bam HI fragment with this sequence:

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5' GAATTCGGTTAATACGACTCACTATAGGGAGATAGGGGGATCC  3'
       |        |        |        |
  EcoRI -20      -10      +1      +10 BamHI
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When this fragment was cloned into M13 mp8 it was possible to use a biological screen for finding mutations in the promoter sequence (3). The pKCT7P-like plasmids contain the galactokinase gene under the exclusive transcriptional control of either the standard or a mutated late promoter (3). A listing of the point mutations studied is shown in table 4.

Footprinting Reactions and Data Analysis

For the footprinting studies, DNA fragments containing either the standard or a mutated late promoter and having a ^{32}P label on one of the 5' ends were prepared as described previously (9).

Methidiumpropyl-EDTA Fe(II) (MPE-Fe(II)) was used as the DNA cleaving agent because it cleaves DNA with little or no sequence specificity and thus reveals protected regions with well defined boundaries (10). The standard

footprinting reaction conditions were: 10mM NaCl, 10mM Tris-HCl pH 7.4, 2mM $MgCl_2$ and 1 - 10 ng of end-labelled DNA restriction fragment. The MPE·Fe(II) was kept reduced by using 1mM ascorbic acid and 4mM dithiothreitol. Addition of T7 RNA polymerase (or polymerase storage buffer) introduced 0.008mM EDTA, 0.08mM NaN_3 and 4% glycerol. When 0.4mM GTP was used, the [$MgCl_2$] was increased to 5mM. T7 RNA polymerase was preincubated with the DNA for six minutes at 37^0 C, then the cleaving reaction was initiated by adding MPE·Fe(II). The reactions were stopped after 5-7 minutes by adding EDTA and tRNA then quickly precipitating with ethanol (9). Cleavage products were resolved by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. The degree of occupancy of the protected base pairs was determined as described earlier (9,11). The position of the footprint was aligned with the sequence of the promoter bearing fragment by using a Maxam-Gilbert style sequencing ladder and a restriction fragment as size markers.

Chemicals and Enzymes

Methidiumpropyl-EDTA was provided by P. B. Dervan (California Institute of Technology). $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was purchased from Baker and Adamson, gamma ^{32}P-ATP from New England Nuclear, and ultrapure GTP from P-L Biochemicals. Yeast tRNA (grade VI from Sigma) was further purified by extracting three times with buffer equilibrated phenol, once with chloroform, twice with ether, then ethanol precipitating twice, followed by treatment with diethylpyrocarbonate. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs or Boehringer Mannheim. T7 RNA polymerase was purified from an overproducing strain as described previously (3,8).

Deletion of Promoter Sequences Upstream from -12

The plasmid pRW370 contains a partially active mutated promoter that differs from the wildtype consensus sequence at positions -11, -13 and -14 (7). An Alu I site at -12 was used to make a deletion that altered the base pairs from -15 to -19 to a sequence having no homology to the T7 late promoter consensus (Table 1). A 115 bp Alu I fragment, which contained the right part of the promoter, was isolated from pRW370 and cloned into the Nru I site of pBR322. Standard microscale procedures were used to prepare plasmid DNA for restriction fragment analysis from ampicillin resistant, tetracycline sensitive colonies (12). A clone with the desired insertion was identified and plasmid DNA was purified by CsCl density gradient centrifugation (13). The sequence of the mutated promoter in pRW377 was determined by the method of Maxam and Gilbert (14).

Unless otherwise stated, the *in vitro* transcription activity of the

partially deleted promoter in pRW377 was tested at 37° C in 0.050 ml of 50mM Tris-HCl pH 7.6, 0.1mM dithiothreitol, 10mM $MgCl_2$, 2.5mM spermidine, 2.5% glycerol, 0.4mM each ATP, GTP, CTP and UTP (plus 10 uCi/ml [^{3}H]-UTP), with a template concentration of 0.2mM in terms of nucleotide monomer, and a T7 RNA polymerase concentration of 7.2 nM. The reactions were incubated for 10 minutes, and then a 0.040 ml portion was assayed for incorporation of [^{3}H]-UMP into acid insoluble material (15).

IN VIVO Studies of Promoter Activity - Galactokinase Assays

The use of pKO1 to place the E. coli galK gene under the transcriptional control of a cloned promoter was described by McKenney et al. (16). In order to use galactokinase assays to measure the expression from a T7 late promoter in vivo, it is necessary to have T7 RNA polymerase in the cells which contain the pKCT7P-like plasmids. This was achieved by using the plasmid pGP1-2, which contains the T7 RNA polymerase gene under the transcriptional control of the bacteriophage lambda P_L promoter (17). The synthesis of T7 RNA polymerase is regulated by a heat sensitive lambda repressor. Shifting the culture to 42° C induces the synthesis of T7 RNA polymerase which then transcribes the galK gene. The in vivo experiments were done in CAG1134, a galK$^-$ recA$^-$ derivative of E. coli C600 (18).

Uninduced cultures were grown at 30° C in LB broth plus kanamycin and ampicillin at 100 mg/l each. Mid-log phase cultures (OD_{650} of 0.25-0.30) were induced by shifting to 42° C, and at various timepoints portions were taken and assayed for galactokinase activity (16). When necessary, cultures were diluted in order to achieve a linear rate of galactose phosphate synthesis. The galactokinase activities were normalized to a culture with a final OD_{650} of 1.0. For each assay a portion of the culture was taken and fixed by the addition of formaldehyde to 2% then stored on ice until the OD_{650} could be checked. Assays were done in triplicate with four time points taken from each reaction at 0, 5, 10, and 15 minutes. Most of the mutated promoters were tested with a 1 hour induction. The time course assay was done on the wildtype promoter and on representative type A and type B mutated promoters. The amounts of T7 RNA polymerase and galactokinase protein in the induced cells were measured as follows: For each sample a 1 ml portion of the culture was taken, and the cells were collected by centrifugation. The supernatant was decanted and the cell pellet was resuspended in SDS sample buffer to a final concentration of 20 OD_{650} units/ml and boiled for 10 minutes. Then the proteins from 0.1 OD_{650} unit of sample were separated by SDS-polyacrylamide gel electrophoresis (19). Gels were stained with

Coomassie Brilliant Blue and protein bands were quantitated by scanning densitometry at 550 nm using a Hoeffer GS-300 with GS-350H software package.

RESULTS

Footprinting Studies of T7 Late Promoters With Point Mutations

Figure 1 shows typical footprints caused by protection of the standard or a mutated T7 late promoter by T7 RNA polymerase. The DNA fragments used here were labelled on the 5' end of the + (antisense) strand. Low salt conditions were used (9). The polymerase concentration was 160 nM. These conditions give 88% protection of the standard promoter when no GTP is added. All of the type A mutated promoters give footprints that are similar to the standard, both with and without GTP, whereas none of the type B mutated promoters gives a visible footprint.

The addition of GTP causes an increase in the size of the footprint observed. For the standard promoter without GTP, the footprint extends from -16 to -4 on the + strand and from -17 to -4 on the - strand. With GTP, the footprint extends from -16 to +5 on the + strand and from -17 to +5 on the - strand, thus covering the +1 initiation site (9,11).

Previous studies gave a binding constant for the interaction of the polymerase with the standard promoter of $(4.6 \pm 1.5) \times 10^7\ M^{-1}$, under low salt conditions (10mM NaCl, 2mM $MgCl_2$) without GTP. Titrations with T7 RNA polymerase, using concentrations between 10 and 100 nM, were used to determine binding constants. These polymerase titrations showed that binding constants for the interactions of the polymerase and type A mutated promoters are indistinguishable from those determined with wildtype promoters (data not shown). However, because no footprints were observed with the type B mutated promoters we estimate that the binding constants for the interaction of the polymerase with type B mutants must be less than $6 \times 10^6\ M^{-1}$, given the sensitivity of the assay.

The type A mutants that have been tested by quantitative footprinting are: +1GT, -2TA, -4TG, -4TA, -6AT, -3AG, -3AC, -3AT, and -5CG. The type B mutants that were tested are: -5CT, -7CG, -8TG, -8TA, -8TC, and -9CT.

Figure 2 shows footprint profiles from typical protection experiments with T7 RNA polymerase. A footprint profile is a plot of the degree of protection or occupancy observed at each base within the protected region. A base is considered to be in the protected region if the observed occupancy is higher than the average of the unprotected region by 3 times the standard deviation of the data from the unprotected region. The occupancy for the

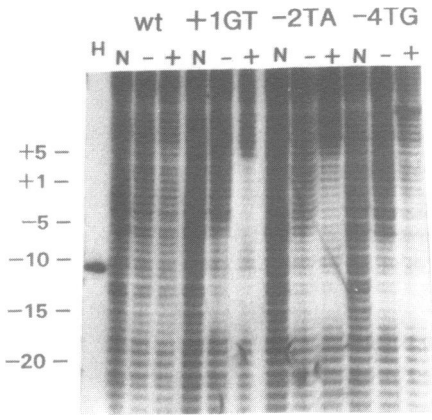

Figure 1
Footprinting the wildtype promoter from pKCT7P and other mutated promoters. Three lanes were run for each promoter tested, the N lanes have no polymerase, the - lanes have 160 nM polymerase and no GTP, the + lanes have 160 nM polymerase with GTP at 0.4 mM. The H lane is a Hinf I digest of the labelled fragment. The wildtype and type A mutated promoters all give similar footprints. The addition of GTP causes an extension of the protected region. The type B mutated promoters produce either no footprints or very weak ones.

entire promoter is determined by taking the average of each site within the protected region. The footprint profile of the interaction between the polymerase and standard promoter in the presence of GTP reflects the extended region of protection and also shows that not all bases within the promoter are protected from cleavage to the same degree. The valley from -12 to -8 indicates a lesser degree of protection at these sites. The profiles of the

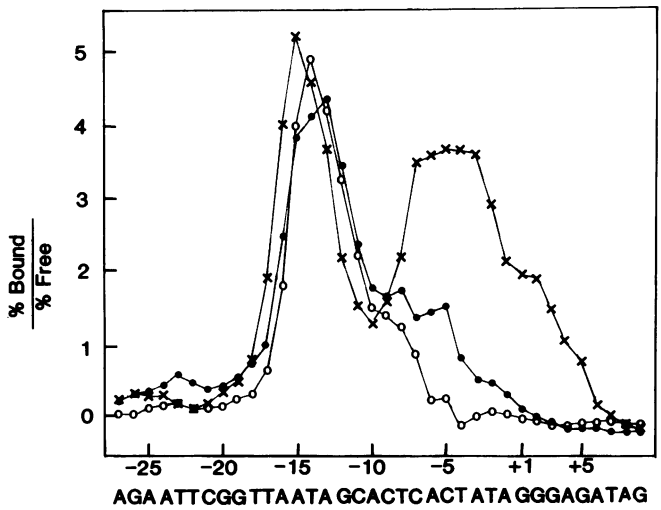

Figure 2
Profiles of the footprints caused by the protection of late promoters by T7 RNA polymerase at a concentration of 96 nM.
Symbols: ● wildtype promoter without GTP, X wildtype promoter with GTP
O -4TG mutated promoter without GTP.

footprints obtained with the type A mutant promoter -4TG and the standard promoter in the absence of GTP show that there is less protection of the bases from -4 to -6 in the mutant. However the overall binding constant is not reduced by the -4TG mutation because the binding to this region of the promoter is relatively weak in comparison to that seen from -16 to -10.

Upstream Boundary of the Sequence Specific Promoter-Polymerase Interaction

Since T7 late promoters are highly conserved, it is natural to presume that the upstream boundary of the sequence specific promoter-polymerase interaction is at the boundary of the conserved sequence. However, it is difficult to define the upstream boundary of the conserved sequence because the class III late promoters show an 84% conservation of GAAAT in the -22 to -18 region but these base pairs are not conserved in the class II promoters. Also, earlier studies indicated that region upstream from -11 was not essential for promoter function in vitro (6,7).

Another promoter with a deletion of the base pairs to the left of -10 has been constructed. Table 1 shows the sequences of the partially deleted promoter in pRW377 and the four similarily altered promoters that have been previously reported. The promoters with deletion mutations can be thought of as having multiple substitutions, because the region of the promoter that is deleted is replaced by another DNA sequence. The mutated promoter in pRW377 differs from the earlier examples in that it has no homology to the T7 late promoter consensus sequence in the region upstream from -10, except for a C at -12. The mutated promoter in pRW377 also differs from the earlier examples in that it is inactive under all conditions tested (it is no longer a promoter). Table 2 shows the results of in vitro transcription studies using pRW377 as a template. The negative and positive controls are, respectively, pKO1 which contains no T7 late promoter (16), and pKCT7P (3). Conditions such as the addition of glycerol, incubation at 42^{o} C, supercoiled templates, and low salt have been shown to enhance the utilization of mutated T7 late promoters (3,7). The partial promoter found in pRW377 is inactive even when these conditions are used.

In an in vitro transcription assay the rate of RNA synthesis depends on many factors in addition to the rate of transcription initiation. The rates of transcription elongation and termination depend in part on the sequence of the template. Since the plasmids pKCT7P, pRW370, pRW371 and pRW377 are different, one cannot make a direct quantitative comparison of their respective initiation rates from the in vitro transcription assays. However, these assays are sufficient to show that the mutated promoter in pRW377 is inactive, whereas pRW370 and pRW371 show some activity.

TABLE 1
PROMOTERS WITH DELETIONS OF THE LEFTWARDS BASE PAIRS

Promoter	Sequence (-20 -10 +1)	Ref.
pRW377	ctggcgttcgCtACTCACTATAGGAGAA	this work
pRW370 [a]	cgcATTAAagCtACTCACTATAGGAGAA	7
pRW371	ccgcaItAaAgcACTCACTATAGGAGAA	7
OC 1 [b]	ttgtccAtIgaGACTCACTATAGGGAGA	6
OC 2 [b]	GtAcaIcAcAaGACTCACTATAGGGAGA	6
Consensus [c]	GAAATTAATACGACTCACTATAGGGAGA	2

a The Alu I site is boxed.
b OC 1 and OC 2 designate the two promoters with deletion mutations that were constructed in vitro by H. Osterman and J. Coleman (6).
c The class III consensus sequence is shown, class II promoters do not show conservation of the -22 to -18 region. In the promoters with deletion mutations, bases identical to the consensus sequence are shown as underlined capital letters.

An examination of the sequences of the 4 active mutant promoters shown in table 1 and the 17 natural T7 late promoters (23) reveals that most of the base pairs in the -22 to -11 region of the pRW377 promoter are found in active promoters. The sole exception is the G at -17. Because there is no example of an active T7 late promoter with a G at -17 it is possible that this G is the key factor in making the pRW377 promoter inactive. Alternatively, the pRW377 promoter could be inactive because it lacks specific base pairs in the region upstream from -11 that could interact favorably with T7 RNA polymerase. The difference between the inactivated promoter in pRW377 and the weak promoter in pRW370 lies in the -14 to -19 region. It is highly probable that the activity of the promoter in pRW370 is in part dependent upon interactions between the polymerase and specific base pairs in the -15 to -19 region that are identical to those found in the class III promoter consensus sequence. The four partially active promoters with deletion mutations all have some base pairs in the -11 to -19 region that are identical to those found in the consensus sequence and these base pairs may be involved in promoter activity. However, these promoters have different

TABLE 2
IN VITRO TRANSCRIPTION ACTIVITY OF PROMOTERS WITH DELETIONS

TEMPLATE		CONDITION		
Plasmid	Promoter Type	std.[a]	12.5% Glycerol	42°C
pKO1	no promoter	-0.2	-0.3	-0.1
pKCT7P	wildtype	100 (1223)[b]	100 (956)[b]	100 (1520)[b]
pRW371	deletion	60	80	63 ± 3 [c]
pRW370	deletion	8.0	8.4	9.7 ± 0.6 [c]
pRW377	deletion	0.3	0.3	0.1

a std. = standard conditions described in Materials and Methods.
b Parenthetical values give the picomoles of ^{3}H-UMP incorporated in a ten min. reaction. Other values are given as % of wildtype.
c Typical error ranges are shown.

sets of wildtype base pairs. This may indicate that all of the base pairs in the -11 to -19 region could interact specifically with the polymerase.

Utilization of Promoters with Point Mutations IN VIVO

The activity of the wildtype or a mutated promoter can be quantitated in vivo by measuring the expression of the galK gene of a pKCT7P-like plasmid. Table 3 shows the results of galactokinase assays using mutated promoters with a polymerase induction time of 1 hour. The activities of the mutated promoters are given as a percentage of the activity seen with the wildtype promoter. A control experiment, using a strain containing pKCT7P and pGP1-2, was included each time a set of mutated promoters was examined in order to compensate for the day-to-day fluctuation in the galK activity observed with a given promoter. The activity of a given mutant relative to the control was fairly consistent from day-to-day. Each reported activity was determined as described in the Materials and Methods section. No galK activity was ever detected in a negative control experiment using a strain that contained pGP1-2 and pKO1. During a 1 hour induction at 42° C, the cells continued to grow with the OD_{650} increasing from about 0.3 to about 0.8. In one hour, type A mutated promoters produce about 70% of the amount of galactokinase as observed when using the wildtype promoter, but type B mutated promoters produce less than 15% of the wildtype level of activity.

Table 3.
GALACTOKINASE ACTIVITY OF CLONES WITH MUTATED T7 LATE PROMOTERS
Shown as a % of the Activity Observed When Using the Wildtype Promoter

Type A Mutations		Type B Mutations	
Mutation	Activity (% wt)	Mutation	Activity (% wt)
-3AC	72 ± 10	-5CT	8 ± 4
-3AT	70 ± 14	-7CT	13.3 *
-3AG	70 ± 15	-7CA	1.8 *
-4TG	104 ± 5	-9CT	3.0 ± 0.4
-4TC	71 *		
-5CG	66 ± 2		
-6AT	74 *		

* one trial only
Typically the wildtype promoter gave incorporations of about 20,000 dpm of C^{14} labelled galactose-phosphate in the sample taken at 15 minutes. The typical error in calculating an activity of a given trial was ± 10%. The induction time for these experiments was 1 hr.

We also examined the effects of various times of induction on in vivo promoter activity. Figure 3 shows a time course of galactokinase synthesis following a shift to 42° C. In a strain with a type A mutated promoter, galactokinase production was slightly less than the wildtype level at all times. In a strain with a type B mutated promoter, galactokinase production was substantially less than the wildtype level at the early timepoints, but at late timepoints the galactokinase activity equalled the wildtype level.

The synthesis of T7 RNA polymerase and galactokinase was monitored by SDS-polyacrylamide gel electrophoresis. Figure 4 shows that the rate of appearance of T7 RNA polymerase did not significantly vary in the strains tested. The synthesis of galactokinase protein parallels the appearance of galactokinase activity (data not shown).

DISCUSSION

Our results indicate that T7 late promoters consist of two functionally different domains. Table 4 shows all of the promoters with point mutations studied and summarizes the results. The footprinting studies on the mutated

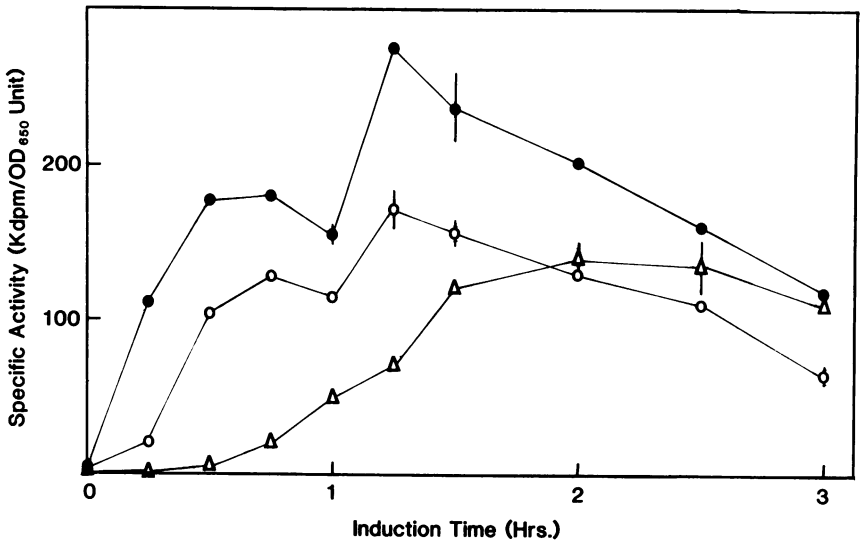

Figure 3
Time course of in vivo expression of galactokinase, using wildtype or mutated T7 late promoters. Symbols: ● wt promoter, O type A mutant promoter -4TG, ▲ type B mutant promoter -8TG.

late promoters show that the type B mutations reduce the binding of polymerase, but that the type A mutations have little effect on binding. Differences seen in the promoter activities of the type B and type A mutants are probably a consequence of a division of promoter functions. The type B and type A mutations map in distinct clusters (3). Point mutations in the -9 to -7 region are all type B, whereas all point mutations downstream from -4 are type A. Both type A and type B mutations are found in the -5 to -6 area. It should also be noted that there is a good correlation between the rightward boundary of the region protected by the polymerase from MPE·Fe(II) cleavage in the absence of GTP, and the boundary between the type B and type A mutations. The type B mutations all lie in the -17 to -4 region which is protected both with and without GTP, whereas most type A mutations lie in the region that is protected only when GTP is present (9).

GTP plays a major role in causing or stabilizing the shift between two types of polymerase-promoter complexes. A previous report showed that in the absence of nucleotides T7 RNA polymerase can form a complex with late promoters that makes the bases from -6 to +4 of the antisense strand sensitive to attack by a single strand specific endonuclease (20). Possibly,

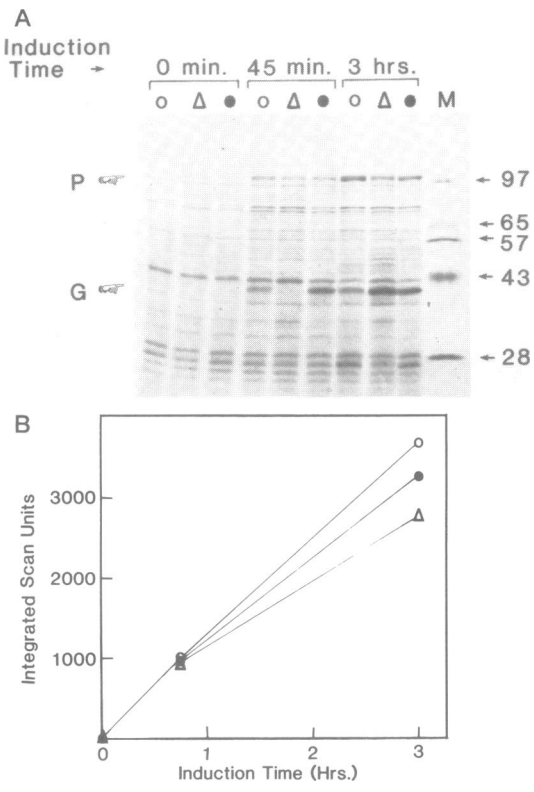

Figure 4
A) SDS-polyacrylamide gel showing the synthesis of T7 RNA polymerase and galactokinase after induction. The symbols are as in Figure 3. The P shows the location of T7 RNA polymerase, and the G shows the location of galactokinase. The sizes of the marker proteins in lane M are shown on the right (kilodaltons).
B) Appearance of T7 RNA polymerase during induction as determined by densitometric scanning of the gel shown in part A. The symbols are as in Figure 3. The cellular concentration of T7 RNA polymerase increases with time and there is no significant difference between the strains tested.

the open complex detected by enzymatic hydrolysis is transient and not stable enough to prevent cleavage by MPE·Fe(II). Although two T7 late promoters are reported to occasionally start chains in vitro with A (23), most late promoters from bacteriophages T7, T3, and SP6 initiate transcription with G (21). If GTP has a role in the initiation process beyond merely being the first nucleotide incorporated, there would be selection pressure to retain a G at +1. One can speculate that during initiation GTP binds to the polymerase in addition to forming a Watson-Crick pairing with the C in the antisense strand.

The in vivo expression data confirm and clarify the observations of earlier in vitro transcription studies (3). The result of the time course in vivo expression experiment is consistent with the idea that type B mutations reduce promoter activity by affecting binding. Early after induction, the T7 RNA polymerase concentration is low and the type B mutated promoters are significantly less active than the standard or type A mutants. Later, when the cellular concentration of polymerase increases, the decreased binding

Table 4

SUMMARY OF DIFFERENCES BETWEEN TYPE A AND TYPE B MUTATIONS

Mutation Type	B	A
Proposed Function	Binding	Initiation
Location	-9 to -5	-6 to +1
Effects on in vitro Transcription		
Supercoiled Template	severely down	no effect (low salt)
Linear Template	severely down	slightly down
Effects on in vivo Expression of Galactokinase		
Short Induction (1 hour) low [polymerase]	severely down	slightly down
Long Induction (3 hours) high [polymerase]	no effect	slightly down
Polymerase Binding (Footprinting)	decreased	no effect
Point Mutations Studied	g g A G a a G C C C t c t T t G T A T	
Wildtype Sequence	TAATACGACTCACTATAGGGAGA -10 +1	

Note: lower case letters denote type B mutants, upper case letters denote type A mutants. The naturally occurring class II promoter from 16.0% of the T7 genome contains the -5CG deviation from the consensus sequence.

affinity of a type B mutant has less effect on the level of gene expression. The complete time course experiment was done with only one type B and one type A mutant, however we have no reason to suspect that the representative mutations chosen were atypical.

The in vivo activities of the mutated vs. standard promoters more closely resemble those seen in in vitro transcription experiments with 60 mM NaCl added rather than that seen when low salt conditions are used (3). This was expected since the in vivo salt concentrations are higher than those used in the low salt in vitro transcription experiments.

We suggest that the -12 to -7 region may be of critical importance for the discrimination between promoter and nonpromoter sites. A key difference between T3 and T7 late promoters lies at position -11 (22), and all of the point mutations made at -9 to -7 reduce promoter activity (3). The footprint profile for the standard promoter shows a valley from -12 to -8 in the presence of GTP, indicating a lesser degree of protection of these sites.

Osterman and Coleman observed that the binding of T7 RNA polymerase to late promoters did not preclude cutting by the restriction enzyme Hinf I at -10 (6). The polymerase may form loose but important contacts with the middle of the promoter, and a mutation here may have deleterious effects on neighboring contact sites.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institutes of Health (CA 07175, CA 23076, GM 30822, GM 07215 and CA 09135) and the National Science Foundation (83-08644).

We thank Stevan Jovanovich for helpful discussions.

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