

# Mapping of single-stranded regions in duplex DNA at the sequence level: Single-strand-specific cytosine methylation in RNA polymerase–promoter complexes

[cytosine N-3 methylation/*lac* promoter/*trp* promoter/*trp-lac* (*tac*) hybrid promoter]

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**ABSTRACT** A method based on the differential rate of cytosine methylation in single- and double-stranded nucleic acids by dimethyl sulfate [Peattie, D. A. & Gilbert, W. (1980) *Proc. Natl. Acad. Sci. USA* 77, 4679–4682] has been developed for probing unpaired cytosines in DNA and DNA–protein complexes at the sequence level. Application of the method to the complexes between *Escherichia coli* RNA polymerase (EC 2.7.7.6) and three related promoters, *lac* UV5, *trp*, and a hybrid promoter *tac* resulting from the fusion of the two, reveals distinct differences in the way RNA polymerase unpairs DNA in these promoters. No single-stranded region is detectable in the complex with the *trp* promoter. For the *lac* UV5 promoter, the cytosines at positions –6, –4, –2, and –1 are in an unpaired region. The same cytosines in the *tac* promoter, which is homologous in sequence to *lac* UV5 in this region, are also found to be single stranded. For the pair of promoters *lac* UV5 and *tac*, the cytosine methylation reaction has also been used to demonstrate the steep temperature dependence of opening of base pairs by RNA polymerase. One striking feature is that the midpoint of this transition for the *tac* promoter is 3°C lower than the corresponding value for *lac* UV5, even though the sequence of the unpaired region in the two promoters is identical.

The predominant structure of cellular DNA is the familiar right-handed double helix in which the bases are paired (1). It is well recognized, however, that unpairing of the bases occurs in a number of biological processes, including replication and transcription. We report here the use of dimethyl sulfate, a reagent that has been widely used in nucleic acid sequence analysis, for the mapping of unpaired regions in DNA or DNA–protein complexes.

Dimethyl sulfate exhibits several well-characterized site-specific reactions. For DNA, methylation at the N-7 of guanine and N-3 of adenine by this reagent is often utilized in chemical sequencing and in probing interactions between DNA and proteins (2, 3). Dimethyl sulfate alkylation at the N-7 of guanine has similar applications in studying RNA (4, 5). This reagent also attacks the N-1 of adenine and the N-3 of cytosine (6). Because these positions are involved in hydrogen bond formation in a Watson–Crick duplex structure, methylation at these sites is much diminished in such double-stranded structures. The single-strand specificity of methylation at N-1 of adenine has been exploited previously by Melnikova *et al.* for the determination of the extent of unpairing of bases induced by RNA polymerase (EC 2.7.7.6) (7). The same reaction has been used by Siebenlist *et al.* to determine the particular region within a promoter that becomes unpaired by the binding of the poly-

merase (8, 9). Because methylation at N-1 prevents the pairing of the base with its complement, upon removal of the bound polymerase the positions of the N-1-methylated adenines can be determined subsequently by the use of single-strand-specific nuclease (8, 9). Siebenlist *et al.* located the region of promoter sequence that is unpaired by *Escherichia coli* RNA polymerase to be base pairs between –10 and +2 or +3, numbered relative to the major start of transcription at +1, for the phage T7 A3 and the *lac* UV5 promoters (8).

The use of methylation at the N-3 position of cytosine to probe single-stranded regions in a nucleic acid is made possible by the observation of Peattie and Gilbert that in RNA N<sup>3</sup>-methylcytosine is more reactive to hydrazine than is cytosine. Therefore, chain cleavage of RNA can be made to occur more readily at the N<sup>3</sup>-methylcytosine sites, and this in turn permits the chemical mapping of unpaired cytosines in an RNA at the nucleotide sequence level (5, 10). This technique has been used to study the temperature-dependent unpairing of specific cytosines in tRNA (5) and the secondary structure of tRNA complexed with ribosomal proteins (11). We have found that the same type of preferential chain cleavage can occur in DNA, and we report here the application of this reaction to the study of the unpairing of bases within a specific region of a promoter by RNA polymerase.

## MATERIALS AND METHODS

*E. coli* RNA polymerase was prepared according to the modified procedure of Burgess and Jendrisak (12). The various promoter-containing restriction fragments, end-labeled at a unique 5' or 3' end, were prepared as described by Maxam and Gilbert (13). Incubations of purified labeled promoter fragments with RNA polymerase were performed in a buffer containing 25 mM Hepes adjusted to pH 8, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and bovine serum albumin at 100 µg/ml. DNA concentrations were approximately 4 nM for the particular restriction fragment, and RNA polymerase was usually 80–200 nM. Incubation was usually 20 min at temperatures of 20°C or lower and 10 min at the higher temperatures. Protection from DNase I (14) was used to check for complex formation in the presence and absence of dimethyl sulfate as described (15). Reactions with dimethyl sulfate were typically performed with 50–200 mM alkylating reagent.

Experiments to detect cytosines in single-stranded regions with dimethyl sulfate and hydrazine (5) were conducted as follows. To each reaction mixture containing end-labeled DNA and RNA polymerase in 50–100 µl of the binding buffer, 1 µl

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of dimethyl sulfate was added. The incubation time was optimized to give the best signal-to-noise ratio for the bands on the sequencing gel that result from cleavage at the positions of the cytosines in the single-stranded region. At 30°C, a few minutes of incubation is usually sufficient; at 15°C, incubation is typically 10 min or longer. The optimal level of methylation is around 2–3 methylated guanines and cytosines per 100 nucleotides.

The DNA reactions were stopped with 200  $\mu$ l of a solution of 3 M ammonium acetate, 1 M 2-mercaptoethanol, tRNA at 100  $\mu$ g/ml, and 20 mM EDTA. The DNA was precipitated with ethanol, washed, and dried. Each pellet was then redissolved in 20  $\mu$ l of H<sub>2</sub>O or 10  $\mu$ l of H<sub>2</sub>O and 10  $\mu$ l of distilled dioxane. The dioxane appears to enhance the differential reactivity to hydrazine between methylated and unmethylated cytosines. The solutions were cooled to 0°C, 20  $\mu$ l of cold hydrazine was added to each, and the mixtures were incubated at 0° for 7 min. The reactions were stopped by the addition of 200  $\mu$ l of cold 0.6 M sodium acetate, and 3 vol of cold ethanol was added to precipitate the DNA. The samples were redissolved in 1 M piperidine, heated at 90°C for 30 min, and lyophilized (13). The lyophilized material was finally resuspended in formamide loading buffer and electrophoresed through thin 50% urea sequencing gels (16).

## RESULTS

**Methylation at N-3 of Cytosine by Dimethyl Sulfate Can Be Used to Map Single-Stranded Regions in DNA at the Sequence Level.** Fig. 1 illustrates the single-strand-specific methylation of the cytosines by dimethyl sulfate. The DNA used in obtaining the sequence patterns in lanes c and d of this experiment was a uniquely end-labeled single strand obtained by separating the strands of a short restriction fragment containing

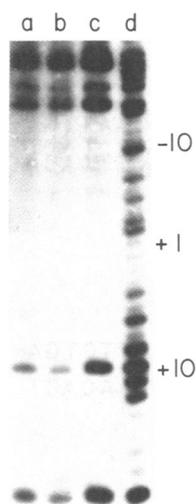


FIG. 1. Sequence analysis of an end-labeled, double-stranded (lanes a and b) or single-stranded (lanes c and d) restriction fragment. The labeled fragment was treated sequentially with dimethyl sulfate and piperidine (lanes a and c) or dimethyl sulfate, hydrazine, and piperidine (lanes b and d). A 203-base-pair DNA fragment containing the *E. coli lac* wild-type promoter was uniquely labeled at the 5' end of the template strand. The labeled strand was isolated in single-stranded form by preparative gel electrophoresis (13). All four samples were incubated in Hepes buffer containing 175 mM dimethyl sulfate for 2 min at 37°C. The samples shown in lanes a and c were precipitated twice and treated with piperidine to cause chain breakage at guanine residues. The samples displayed in lanes b and d were precipitated, treated with hydrazine to modify any methylated cytosines, reprecipitated, and treated with piperidine. Samples were electrophoresed through 50% urea/8% polyacrylamide gels, and the gel was subsequently autoradiographed as described (13).

the wild-type *lac* operon control region. This end-labeled single strand was treated with dimethyl sulfate alone (lane c), or sequentially with dimethyl sulfate and hydrazine (lane d) in the manner described in *Materials and Methods*. These patterns are to be compared with those of lanes a and b, for which the double-stranded restriction fragment containing the end-labeled strand was similarly treated. The bands that are present in lanes a–c are derived solely from cleavages at guanines that are obtained by treatment of the methylated fragments with piperidine under conditions that induce chain cleavage only at the guanines (13). The additional bands in lane d that are seen only with the restriction fragment in the denatured form are due to cleavages at the cytosines. All of the cytosines in this region, at positions –13, –9, –6, –4, –2, –1, +5, +7, +9, +11, and +12, are detected by the N-3 cytosine methylation reaction when the DNA is in the single-stranded form but not when the DNA is in the double-stranded form.

**Four Cytosines of the *lac* UV5 Promoter at Positions –1, –2, –4, and –6 Are in a Single-Stranded Region in the RNA Polymerase–Promoter Complex.** The above experiment demonstrates that methylation at the N-3 position of cytosine can detect single-stranded regions at the sequence level. An additional advantage of this reaction is that it is sufficiently mild to be readily applicable in monitoring the unpairing of DNA at unique sites by proteins or other molecules.

Fig. 2 displays the fragmentation patterns of a restriction fragment containing the *lac* UV5 promoter, with the template strand (the “bottom strand”) end labeled. Lane a is the pattern

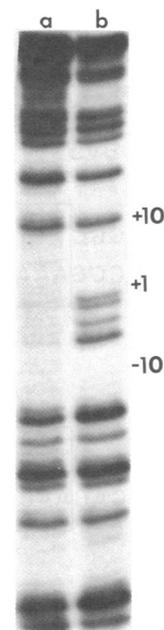


FIG. 2. Electrophoretic patterns of uniquely end-labeled DNA fragments bearing the *lac* UV5 promoter resulting from the stepwise treatment of the DNA fragment with dimethyl sulfate, hydrazine, and piperidine in the absence (lane a) and presence (lane b) of RNA polymerase at 30°C. The double-stranded 95-base-pair fragment was labeled at the 3' end of the template strand; nucleotides are numbered relative to the start of transcription, as in the UV5 sequence given in Fig. 3. DNA fragments at a concentration of 4 nM were incubated in Hepes buffer with 80 nM RNA polymerase for 10 min at 30°C. Dimethyl sulfate was added to 200 mM and incubation was continued for 5 min. Parallel samples were digested before and after dimethyl sulfate treatment with DNase at 0.05  $\mu$ g/ml for 2 min; the resulting patterns of protected fragments (data not shown) guarantee both that the promoter was saturated with bound polymerase and that this complex was not disrupted by the dimethyl sulfate treatment.

obtained with the DNA alone and lane b is that obtained with the DNA-RNA polymerase complex; in both cases methylation was carried out at 30°C and hydrazinolysis was performed under standard conditions. The prominent bands present in both lanes correspond to the guanines in the sequence. The characteristic enhancement of the band at position -14 (17) in the presence of RNA polymerase at the incubation temperature (30°C) provides an internal control that the RNA polymerase-promoter complex is indeed intact under these conditions; this is further confirmed by protection of the complex from DNase I (data not shown).

Treatment of the DNA with hydrazine after the extraction of polymerase and dimethyl sulfate gives rise to the four additional bands seen in lane b. These correspond to the cytosine residues at positions -1, -2, -4, and -6 in the sequence. No bands are seen at +5 or -13, though there are cytosines at these positions in the *lac* UV5 promoter as well. The four cytosines at -1, -2, -4, and -6 are located in the region of DNA that has been shown to be unwound in the presence of polymerase (8, 9). Therefore this protocol, as described in *Materials and Methods*, makes possible the detection of unpaired cytosine residues in DNA molecules complexed with protein.

**Cytosine Methylation Reaction Shows that RNA Polymerase-Induced Unpairing of Bases Is Strongly Dependent on Temperature and Promoter Sequence.** Transcription from a duplex DNA template is strongly temperature dependent. One interpretation of this strong dependence is that the promoter-polymerase complex undergoes a transition from a "closed complex" at low temperature to an "open complex" at high temperature; the transition is characterized by the unpairing of a short segment in the promoter.

The cytosine N-3 methylation reaction offers a way to examine directly the temperature dependence of the RNA poly-

merase-induced opening of base pairs. We have examined the three promoters whose sequences are given in Fig. 3: the *lac* UV5 promoter, the *trp* promoter, and a *trp-lac* hybrid promoter, termed *tac*, that was constructed by joining the -35 region of the *trp* promoter to the -10 region of the *lac* UV5 promoter (19, 20).

For the *lac* UV5 promoter, no cytosine methylation is detectable when the promoter-RNA polymerase complex is treated with dimethyl sulfate at a temperature of 15°C or lower. In Fig. 4 the average intensity of the characteristic quartet on the sequencing gel that results from methylation of the cytosines at positions -1, -2, -4, and -6 (see Fig. 2) is plotted as a function of the temperature of the methylation reaction: it reaches its half-maximal value around 22.5°C and approaches its maximal value around 25°C. We detect no differential temperature dependence of the intensities of the individual bands of the quartet, and therefore only the average value is plotted here.

Because the -10 regions of the *lac* UV5 and *tac* promoters are identical, it is not surprising that a similar quartet resulting from methylation of the corresponding cytosines in the *tac* -10 region is seen with the *tac* promoter. There are, however, significant differences between the two promoter complexes. The temperature at which half-maximal intensity is reached for the *tac* complex is about 3°C lower than that of the *lac* UV5 complex. The quartets also differ in their intensity distributions. In the *lac* UV5 case, the band corresponding to cleavage at the cytosine at -6 is the most intense; in the *tac* case, it is the band corresponding to cleavage at the cytosine at -4.

Additional differences between the *lac* UV5 and *tac* complexes with RNA polymerase at 30°C are revealed by the guanine methylation patterns. Some characteristics of N-7 methylation of the guanines in the promoter-polymerase complexes are indicated in Fig. 3. The guanine residue in the *tac* pro-

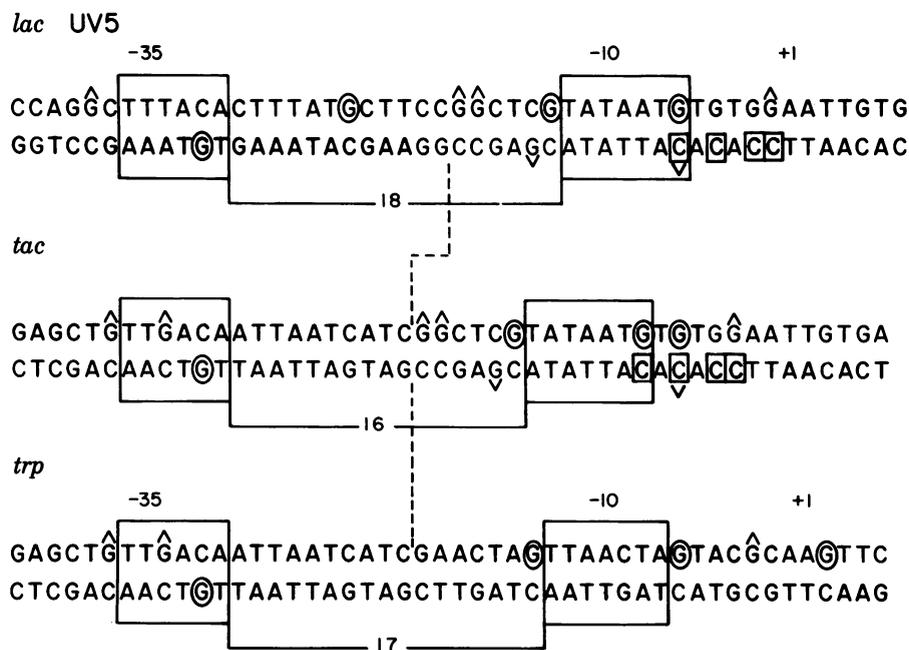


FIG. 3. Nucleotide sequences of *E. coli trp* (18), *lac* UV5 (17), and *tac* hybrid (19, 20) promoters. The promoter sequences are oriented so that transcription is from left to right; the bottom strand as written is the template strand. The starting nucleotides of transcription for the *trp* and *lac* UV5 promoters are assigned a position of +1 in the sequence. For the *tac* promoter, the transcription start is not yet known. We have used in the text the same numeration as in *lac* UV5 for the sequences that are common to both. Boxed areas indicate -35 and -10 regions of homology between promoters (21), and the distance between these regions (in base pairs) is given for each promoter. The broken vertical lines show the junction between *trp*- and *lac* UV5-derived sequences in the *tac* promoter. Circles and carets indicate those guanines whose reactivity to dimethyl sulfate is diminished or enhanced, respectively, in the presence of bound RNA polymerase (data not shown; also refs. 3, 17, and 22). Small squares mark the cytosines that become single-stranded in the presence of RNA polymerase; carets indicate that cytosine in each sequence that is most reactive toward dimethyl sulfate when RNA polymerase is bound.

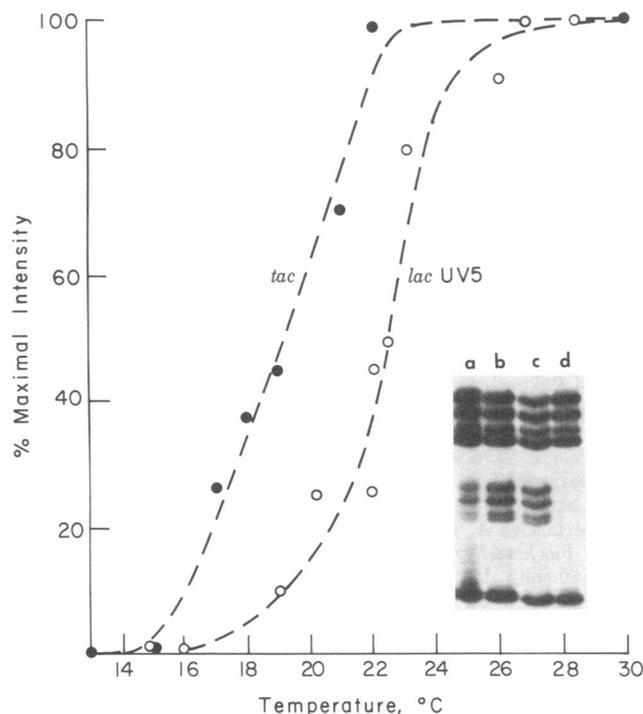


FIG. 4. Temperature dependence of cytosine methylation in the presence of RNA polymerase for *lac* UV5 and *tac* promoters. The extent of methylation of the cytosines in the  $-10$  region of the *tac* (●) and *lac* UV5 (○) promoters by dimethyl sulfate in the presence of bound RNA polymerase was measured as a function of incubation temperature. At each temperature, saturation of the promoter by bound RNA polymerase was verified by DNase I protection. The length of incubation of RNA polymerase–promoter complexes with 200 mM dimethyl sulfate was varied from 5 min at 30°C to 20 min at 10°C in order to achieve approximately the same extent of methylation for each sample. Hydrazine and piperidine treatments and sequencing gel electrophoresis were performed identically for each sample. The resulting autoradiograms were scanned with a densitometer. The extent of cytosine methylation in each sample was determined by normalizing the average band intensities from the four cytosines at positions  $-1$ ,  $-2$ ,  $-4$ , and  $-6$  to the band intensities from guanine residues represented by fragments both longer and shorter on the sequencing gel (guanines at  $+17$ ,  $-16$ ,  $-19$ , or  $-20$ ). Values for the extent of cytosine methylation in the presence of RNA polymerase below 15°C were indistinguishable from controls in the absence of the enzyme for both promoters: this value was defined as 0% maximal intensity on the graph. For each promoter, there was a temperature beyond which the calculated extent of cytosine methylation no longer increased; the value at this point was defined as 100% maximal intensity. Intermediate values were normalized to a linear scale within this 0–100% range; the error for each point is estimated to be about 10%. For the *lac* UV5 promoter, this curve is unaffected by incubating the RNA polymerase–promoter complex at 37°C before incubating at the given temperature. (Inset) Portion of a sequencing gel displaying some of the data from which this graph was derived. A 400-base-pair fragment bearing the *tac* promoter (19, 20), uniquely end-labeled at the 3' end of the template strand, was incubated at 18°C (lane a), 22°C (lane b), and 30°C (lanes c and d) in the absence (lane d) and presence (lanes a, b, and c) of saturating amounts of RNA polymerase. Incubations were continued in the presence of 200 mM dimethyl sulfate for 6, 4, and 2 min for the 18°C, 22°C, and 30°C reactions.

moter–polymerase complex corresponding to the guanine residue at position  $-4$  of the *lac* UV5 promoter is notably better protected from dimethyl sulfate, suggesting the presence of an additional promoter–polymerase contact in the *tac* complex that is absent from the *lac* UV5 complex.

For the *trp* promoter, although several cytosines are present in the  $-10$  region (at positions  $+3$ ,  $-2$ ,  $-6$ , and  $-14$  in the template or bottom strand and at positions  $-2$  and  $-9$  in the

top strand), we could not detect methylation of any of these cytosines at temperatures as high as 37°C. The trivial interpretation that the *trp* promoter does not form a unique complex with RNA polymerase in our experiments is made highly unlikely by the characteristic protection and enhancement patterns of the guanines (Fig. 3 and ref. 22) and by DNase I protection (data not shown). Indeed, all polymerase contacts revealed by the guanine methylation and DNase I cleavage patterns of the *tac* and *trp* promoters are identical in the  $-35$  region, which is reasonable in view of the sequence identity of the two promoters in this region.

## DISCUSSION

The results presented above demonstrate the applicability of the cytosine N-3 methylation reaction in probing the single-stranded regions in a duplex DNA. The signal-to-noise ratio of this method for detecting unpaired cytosines is limited by two major factors. The reactivity of unmethylated cytosine with hydrazine is appreciable; the resulting chain cleavages at the positions of the unmethylated cytosines therefore give a significant level of background. Also, the principle of sequencing an end-labeled linear polymer by chain breakage requires the introduction of no more than a few breaks within a distance from the labeled end. This distance is determined by the resolving power of the sizing method, typically a few hundred nucleotides for nucleic acids. Because the guanines are readily methylated, and chain breakage by piperidine occurs at the positions of the N-7-methylated guanines on a sequencing gel the maximal intensity of the bands resulting from methylation of the cytosines is about one-third of the average intensity of the bands resulting from the methylation of the guanines (see Fig. 1). These limitations on the signal and the noise tend to narrow the window for optimization of the reaction conditions; in cases in which the cytosines are unpaired only a small fraction of the time, the method is unlikely to give an unequivocal answer.

Nevertheless, the mildness of the methylation reaction and the utility of the cytosine methylation method in mapping unpaired cytosines at the sequence level are distinct advantages. The method complements others using single-strand-specific nucleases or a combination of chemical and nuclease treatments (7, 23), in terms of both selecting reaction conditions and avoiding artifacts that might be introduced by the use of structural probes. In addition, the cleavage of the DNA chain at the N<sup>3</sup>-methylcytosine positions is relatively insensitive to the nucleotide sequence, compared to the sequence dependence of cleavages by enzymes.

Our studies on the RNA polymerase–promoter complexes demonstrate the applicability of the cytosine methylation reaction to probing the positions of unpaired cytosines in protein–DNA complexes and add further evidence supporting the RNA polymerase-induced unpairing of bases in a specific region of the promoters (8, 9).

The sharp temperature dependence of the unpairing of the cytosines in the *lac* UV5 and *tac* promoter complexes of RNA polymerase suggests that the strong temperature dependence of the formation of an initiation complex, in which RNA chain initiation occurs, is closely related to the RNA polymerase-induced unpairing of bases in the promoter. The three promoters we examined show rather distinct differences in their uncoiling by the polymerase. Whereas unpairing of bases in the *lac* UV5 and *tac* promoters is readily detected by cytosine methylation, no cytosine in either strand of the *trp* is made reactive to dimethyl sulfate by polymerase binding.

The  $-35$  region of the *trp* promoter is identical in sequence to the  $-35$  region of the *tac* promoter and corresponds to the canonical sequence for this region deduced from a compilation

of promoter sequences (21). The contacts with the polymerase in the  $-35$  region, as revealed by probing of the guanines with dimethyl sulfate, are identical for the *trp* and *tac* promoters (see Fig. 3). For the *Salmonella typhimurium trp* promoter, which shows extensive homology with the *E. coli trp* promoter, it has been reported that the  $-35$  and  $-10$  regions act as separate domains in their interactions with the polymerase: mutations in the  $-10$  region that diminish the RNA polymerase-mediated protection of the promoter against cleavage at position  $-11/-12$  by the restriction enzyme *HincII* do not affect the protection of the promoter against cleavage at position  $-34/-35$  by the same restriction enzyme (24). For the pair of related promoters *trp* and *tac*, the  $-35$  region sequence that is the same for both presumably determines the positioning of the RNA polymerase in each case. The large difference between the two promoters with respect to their unwinding by the polymerase must be primarily due to the difference in their sequences in the  $-10$  region.

The apparent inaccessibility of the cytosines in the  $-10$  region of the *trp* promoter suggests that this promoter forms an open complex only transiently. An alternative, and we believe less attractive, interpretation is that the cytosines are unpaired but they are blocked from methylation by their interactions with the RNA polymerase. For a number of promoters, selective transcription occurs readily, yet no open complex is detectable by criteria such as the stability of the complex and its resistance to attack by polyanionic inhibitors (25). The *trp* promoter is likely to fall in this category; it should be interesting to examine more promoters of this class by the cytosine methylation method. Parenthetically, we note that transcription from the *trp* promoter *in vivo* appears to be insensitive to the degree of supercoiling of the DNA (26, 27). Whether this lack of a supercoiling effect is related to the transiency of the open promoter complex is unclear.

For the pair of promoters *lac UV5* and *tac*, the sequences in the  $-10$  region are identical, and comprise a canonical "Pribnow box." There are clearly differences, however, in the way these  $-10$  region sequences interact with polymerase. The most striking difference is that the *tac* promoter is unpaired by RNA polymerase at a lower temperature than is *lac UV5*. *tac* appears to be the stronger promoter *in vivo* (19, 20); the relationship between these observations is unclear at present. For the related promoters *tac* and *lac UV5*, the way RNA polymerase interacts with the upstream sequences (derived from *trp* in one case and *lac* in the other) has a strong influence on its pattern of interaction with the same downstream sequence. This could be due to differences in the  $-35$  region sequences themselves as well as the change in spacing between the canonical  $-35$  and  $-10$  regions. There is evidence that this spacing affects open complex formation (28). There is, in addition, the possibility that this difference in spacing may force the RNA polymerase

to contact a Pribnow box in the *tac* promoter that is shifted from the one used in *lac UV5*.

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