In Vitro Characterization of Hybrid Promoters and Altered Tryptophan Operon Promoters†

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ABSTRACT: This study examines the in vitro interaction of hybrid and altered *Escherichia coli* promoters and other promoters with purified *E. coli* RNA polymerase. Three parameters of polymerase activity were examined: the time for open complex formation; the temperature of transition; and the time required for productive initiation. The results indicate the rate of in vitro binding as measured by the filter binding technique does not completely correlate with the in vivo activities among these diverse promoters. Transition temperatures ranged from 13 to 27 °C with the lowest transition temperatures associated with the relatively weak in vivo β-lactamase and anti-*tet* promoters. The productive initiation studies showed a dependence on labeled nucleoside triphosphate concentration when that nucleotide was present early and frequently in the transcript. Promoters containing the −10 region of the *lac* promoter had slow productive initiation rates while *trp* −10 promoter derivatives were generally very fast. In the promoters studied here, a trend was noted between the binding rate and transition temperature studies in that the promoters with the lower transition temperatures tended to bind more rapidly.

Many in vitro promoter binding and transcription studies have been described in the literature. However, due to the lack of a standardized in vivo assay system, only a few attempts have been made to compare in vitro and in vivo measurements from the same set of promoters. In this report, three in vitro parameters have been measured for several promoters described earlier (Russell & Bennett, 1982a,b). These studies have been carried out in an attempt to determine if these in vitro measurements reflect the promoter expression observed with in vivo assays. The characteristics measured here are the rate of formation of stable filter-bound open complexes, the effect of temperature on formation of these complexes, and the rate of productive initiation from preformed complexes.

The binding process is thought to consist of the RNA polymerase binding to the promoter DNA to form an initial closed complex. The stability of the closed complex appears to differ among promoters. For example, the *lac* promoter closed complex appears to be much less stable than a λ promoter closed complex (Stefano & Gralla, 1982a; Hawley & McClure, 1980). The closed complex can then either dissociate or isomerize to form a stable, open complex.

The formation of the open complex appears to be sensitive to the temperature of incubation. It has been suggested that this temperature dependence is due to the RNA polymerase mediated melting of the promoter DNA during formation of the open complex (Stahl & Chamberlin, 1977). Siebenlist (1979) has shown a segment of about 10 base pairs (bp) of promoter DNA is single stranded in the open complex, and Kirkegaard et al. (1983) have studied the opening of this DNA segment as temperature was varied.

The next step of initiation considered here is productive initiation which measures the time required for the open complex to bind appropriate NTPs and produce a ternary complex which initiates and forms a full-length RNA (Stefano & Gralla, 1979, 1980). Munson & Reznikoff (1981) have shown an inverse relationship between the levels of productive and abortive initiation. They have further shown that different promoters produce abortive initiation products in characteristic quantities and sizes. Carpousis et al. (1982) have further shown there is an inverse relationship between the rates of open complex formation and productive initiation for a series of *lac* promoter mutants.

Experimental Procedures

Materials. Escherichia coli RNA polymerase was prepared by the procedure of Lowe et al. (1979). The preparation of *Bsp*RI, an isochizomer of *Hae*III, has been described (Sumner & Bennett, 1981). All other enzymes were purchased from commercial sources. The plasmids containing the promoters used in these experiments have been described elsewhere (Russell & Bennett, 1982a,b; Russell et al., 1984). Preparative amounts of plasmid DNA were isolated as previously described (Sumner & Bennett, 1981). Nitrocellulose BA85 filters (from Schleicher & Schuell, 2.5 cm) were pretreated by soaking in 1 N KOH for 10 min, washing extensively in water, and soaking in binding buffer until use (Oppenheim & Yanofsky, 1980).

DNA Fragment Preparation. The filter binding experiments described have used labeled and unlabeled promoter restriction fragments. To prepare labeled fragments, the plasmid DNA was digested with the appropriate restriction enzymes and then end labeled by using either polynucleotide kinase and [γ-^{32}P]ATP or DNA polymerase I large fragment and [α-^{32}P]-dNTPs. After being labeled, the promoter fragment was isolated from a polyacrylamide gel and purified (Maxam & Gilbert, 1977).

Trp-lac, *tet-lac*, and *lac*UV5 promoter fragments were labeled at *Hin*fI (−340) and *Bam*HI (+36) relative to the promoter. *Trp* promoter fragment from pDR210 was labeled at *Hin*fI ends (−341 and +166); the *lac-trp* promoter was labeled at *Hin*dIII (−95) and *Hin*fI (+37); *tet-trp* promoter was labeled at *Hin*dIII (−77) and *Bsp*RI (+263); the *trp* promoter fragment from pDR702 was labeled at *Bsp*RI (−72) and *Msp*I (+187). A nonpromoter-containing fragment (194 bp) was end labeled and used as a control for nonspecific binding.

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Under the standard conditions used here, less than 5% of nonspecific fragment was bound by RNA polymerase. The same conditions typically allowed binding of 40–60% of the promoter-containing fragments.

Unlabeled DNA for binding experiments was prepared by digesting the plasmid DNA with *Bsp*RI, followed by phenol extraction and ethanol precipitation. The mixture of *Bsp*RI fragments was then used in binding experiments.

Filter Binding Experiments Using ^{32}P-End-Labeled DNA Fragments. The binding mixture contained "binding buffer" [100 mM *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (Hepes), pH 7.4, 10 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol, and 100 mM KCl] and DNA. The level of RNA polymerase required to saturate DNA fragments was determined empirically. All experiments used a standardized RNA polymerase/DNA ratio in excess of this saturating amount. For most experiments, a 5-fold molar excess of enzyme to promoter was used. Binding reactions with rapidly binding promoters (those in Table II with a $t_{50\%}$ value of less than 1 min) contained RNA polymerase at a nominal concentration of 20 nM. For the experiments with the slower binding *trp* promoter fragments, aliquots of the same RNA polymerase preparation were added for a nominal RNA polymerase concentration of 80 nM. In comparison of the RNA polymerase concentrations used in various reports and in different preparations, differences in the proportion of active RNA polymerase molecules must be considered as well as the difficulty in retaining full activity when the RNA polymerase is placed into a reaction mixture (Williams & Chamberlin, 1977).

Association of RNA polymerase–DNA complexes was measured by adding RNA polymerase to a DNA binding mix (330 μL) preincubated at 37 °C. At various times after addition, 30-μL aliquots were removed and added to 170 μL of a binding stop mix containing binding buffer + 100 μg/mL heparin. After 1-min incubation, 180 μL of the mixture was filtered with gentle suction through nitrocellulose filters and washed with 1 mL of binding buffer. The filter was then dried and counted in a toluene-based scintillation cocktail. A background blank taken before addition of RNA polymerase ($t = 0$) was subtracted. Total DNA input was measured by spotting 27 μL of the DNA binding mix on a filter or by spotting 180 mL of binding mix plus stop mix on a filter and counting. The background at $t = 0$ was typically <7% of total input. All results are averages of several experiments and are plotted as the percent of DNA bound at 10 min.

Filter Binding Using Unlabeled DNA Fragments. Plasmid DNA to be bound was cleaved with *Bsp*RI, and 8 μg of each was diluted to 450 μL in binding buffer. The sample was then preincubated at 37 °C. Forty microliters of 2.3 mg/mL RNA polymerase holoenzyme was added, and 50 μL of the mixture was then removed at various times to 250 μL of stop mix. The mixture was filtered through nitrocellulose and washed with 2 mL of binding buffer. The DNA trapped on the filter was eluted by incubating the filter in 0.4 mL of 0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1% sodium dodecyl sulfate, and 0.1 mM EDTA + 50 μg of tRNA for 1 h. The DNA was then ethanol precipitated, dried, resuspended in 25 mL of TE [10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.9, and 0.1 mM EDTA], and electrophoresed on a 5% polyacrylamide gel. The ethidium bromide stained, ultraviolet-illuminated gel was photographed with Poloroid Type 55 Pos./Neg. film. The negative was scanned with a Kratos SD3000 densitometer equipped with an HP 3390A integrator. Percentages are the average of multiple scans as the ratio of DNA bound at various time points to DNA bound at 10 min.

Temperature Binding Assays. Binding assays at different temperatures were carried out by equilibrating 30 μL of binding buffer plus DNA at various temperatures for at least 10 min and then adding RNA polymerase and incubating an additional 10 min; 170 μL of stop mix at room temperature was added and incubated 1 min at room temperature, and then 180 μL was filtered as above.

Productive Initiation. Productive initiation was measured by following the rate of initiation from preformed RNA polymerase–promoter complexes as described by Gralla et al. (1980). Plasmid DNA containing the promoter to be assayed was cleaved with *Bsp*RI or *Bsp*RI + *Hin*fI, phenol extracted, ethanol precipitated, and resuspended in TE. Complexes were formed by adding 25 pmol of RNA polymerase to 1 pmol of cleaved plasmid DNA in 50 μL of binding buffer (100 mM Hepes, pH 7.4, 10 mM $MgCl_4$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 100 mM KCl). Each pKO derivative plasmid contains at least five different promoters: *bla* (Russell & Bennett, 1981), a cAMP–cyclic AMP receptor protein (CRP)-dependent promoter mapped by Queen & Rosenberg (1981), two promoters in the origin of replication (Selzer et al., 1983), and the promoter to be analyzed which is inserted in front of the *gal*K gene). Therefore, the polymerase/promoter ratio was about 5/1. After incubation for 10 min at 37 °C, 50 μL of "initiation mixture" (240 μM ATP, 240 μM GTP, 240 μM UTP, 5–50 μM CTP, 0.2 μCi/mL [α-^{32}P]CTP, and 200 μg/mL heparin in binding buffer) was added to the binding mixture. At various times after addition of the initiation mixture, 10 μL was removed and mixed with 10 mL of termination mixture (140 μM each of ATP, GTP, and UTP, 5–50 μM CTP, 0.1 μCi/reaction CTP, and 400 μM rifampicin in "Binding buffer"). After an additional 15 min at 37 °C, the elongation was stopped by adding 20 mL of saturated urea containing 0.05% bromophenol blue and xylene cyanol and freezing immediately. The sample was heated at 65 °C for 1 min and loaded onto an 8% polyacrylamide–7 M urea gel, and 1000 V was applied for about 6 h. After autoradiography, the RNA bands were excised and counted or the intensity of the band was estimated by scanning densitometry.

Results

Filter Binding Experiments. The purified end-labeled promoter-containing DNA fragments are shown in Table I. Table I shows that only 3% of the nonpromoter fragment could be bound using a very large excess of RNA polymerase holoenzyme. In addition, the *lac-trp* promoter, which produces no detectable *gal*K expression in vivo, only binds to a level of 5%. All of the promoters shown to be active in vivo bound to a level of at least 35% under similar conditions.

One feature common to all filter binding experiments is the observation that the protein–DNA complex will be retained with less than complete efficiency. A certain percentage of the DNA will not be trapped on the filter even at very high levels of protein. The levels of saturation observed here (35–65%) are similar to levels of RNA polymerase–promoter binding reported elsewhere.

Rate of Open Complex Formation. Preliminary experiments and previous reports on the binding rates of the *bla* promoter (Russell & Bennett, 1981) the *lac*UV5 promoter (Maquat & Reznikoff, 1978; Stefano & Gralla, 1979, 1980), and the *trp* promoter ($t_{50\%}$) of 2 min; Oppenheim & Yanofsky, 1980) suggested the *bla* and *lac*UV5 promoters were faster binding promoters than the *trp* promoter. The dependence of the binding rate of *lac* promoters upon the RNA polymerase

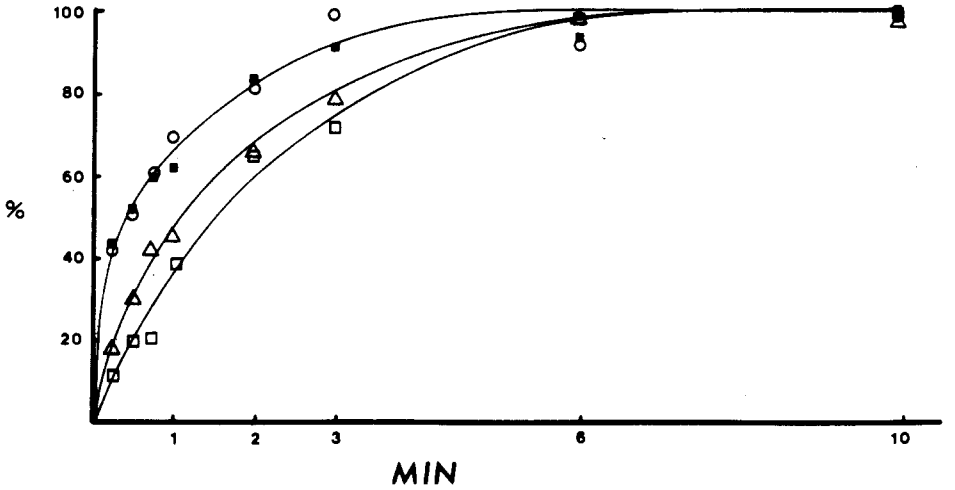

FIGURE 1: Open complex formation of hybrid promoters. The rate of open complex formation is plotted as a percent of filter-bound complexes at 10 min. Curves are mean of multiple determinations. Circles indicate *trp-lac* promoter; triangles *tet-lac* promoter; open squares, *trp* promoter pDR210; closed squares, *lac*UV5 promoter.

Table I: Fragments Used in Binding Experiments

promoter[a]	plasmid source[a]	upstream end	downstream end	fragment size (bp)	% bound at saturation[b]
trp-lac	pDR540	*Hinf* −341	*Bam*HI +36	370	55
tet-lac	pDR209	*Hinf* −341	*Bam*HI +36	370	55
*lac*UV5	pDR206	*Hinf* −341	*Bam*HI +36	370	65
trp (pDR210)	pDR210	*Hinf* −341	*Hinf* +166	500	45
lac-trp	pDR523	*Hin*dIII −95	*Hinf* +37	130	5
nonspecific (no promoter)		*Bam*HI +36	*Hinf* +230	194	3
tet (17-bp spacing)	pDR283	*Bsp*RI −63	*Msp*I +118	177	60
tet (19-bp spacing), anti-*tet*	pDR297	*Bsp*RI −65	*Msp*I +118	179	60
tet-trp	pDR250	*Hind*III −77	*Bsp*RI +263	340	35
trp	pDR702	*Bsp*RI −72	*Msp*I +187	259	45
bla	pBR322	*Bsp*RI −155	*Taq*I −172	327	65

[a] The promoter constructions and plasmids are described in Russell & Bennett (1982b). [b] The percent of the total input counts which were trapped on the nitrocellulose filter after 10 min in the RNA polymerase binding reaction.

Table II

promoter	$t_{50\%}$, open complex (s)	transition temp (°C)	$t_{50\%}$, productive initiation (s)	in vivo[c] promoter activity (galK units)
bla	10–20	13	<15	100
anti-*tet*	10–20	13	ND[d]	70
*lac*UV5	20–30	19	60	510
tet-trp	20–30	23[a]	<15	190
trp-lac	20–30	24	45	1800
tet-lac	40–50	27	45	480
trp (pDR702)	60–70	23	<15[b]	840
trp (pDR702-21)	140–160	23	30[b]	1650
trp (pDR702-23)	160–180	24	<15[b]	1050
trp (pDR210)	90–100	27	<15	1420

[a] *Tet-trp* showed unusually high levels of binding at low temperatures; the transition temperature is taken from the region of the transition rather than at the position of 50% bound. [b] At 20–50 μM CTP. [c] In vivo expression data (Russell & Bennett, 1982b). [d] ND, not determined.

concentration was emphasized by Stefano & Gralla (1980). An effect of RNA polymerase concentration was observed here in some experiments where promoter fragments were subjected to binding under conditions of 2-fold more or 2-fold less RNA polymerase. These experiments did not suggest any unusually sensitive dependence on concentration of the *trp* or *bla* promoter, with the *trp* promoter still binding much slower than the *bla* promoter, and support the general order of binding rates presented in Table II.

Figure 1 shows the rate of complex formation of *lac*UV5-derived promoters and the *trp* promoter. The rapid rates of binding of *lac*UV5 and *trp-lac* promoters did not allow a distinction to be made in this experiment; however, the less active *tet-lac* promoter demonstrated slower binding, with the *trp* promoter even slower.

In Figure 2, two *trp* promoters are compared; one from pDR210 is an essentially normal *trp* promoter, and the other from pDR250 is a hybird made from the −35 region of the pBR322 *tet* promoter and the −10 region of the *trp* promoter. Previous studies (Russell & Bennett, 1982b) and subsequent studies (E. A. Auger, unpublished results) have shown this *tet-trp* promoter to have lower expression than the *trp* promoter in analogous plasmids where both would transcribe the same

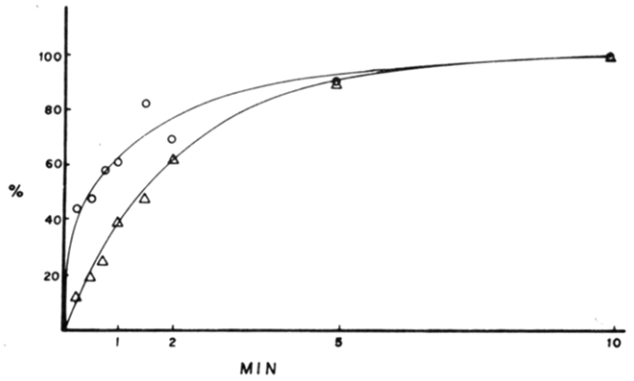

FIGURE 2: Open complex formation of *trp* and *tet-trp* promoters. The rate of open complex formation is plotted as a percent of filter-bound complexes at 10 min. Curves are mean of multiple determinations using end-labeled fragments and by visualizing bound DNA with gel electrophoresis and ethidium bromide staining. Circles, *tet-trp* promoter; triangles, *trp* promoter pDR210.

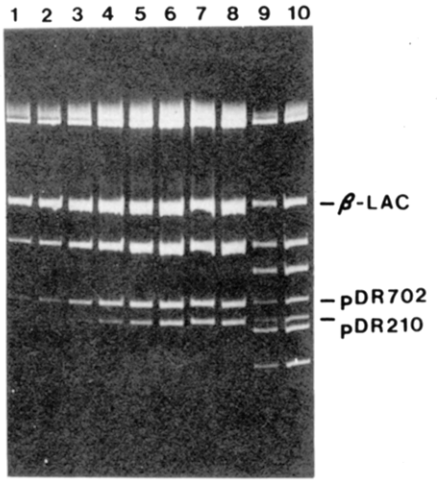

FIGURE 3: Electrophoresis of pDR210 and pDR702. pDR210 and pDR702 were mixed and digested with *Bsp*RI and then subjected to filter binding as described under Experimental Procedures. Binding was stopped at various times and loaded into lanes as indicated: lane 1, 10 s; lane 2, 20 s; lane 3, 30 s; lane 4, 1 min; lane 5, 2 min; lane 6, 5 min; lane 7, 10 min; lane 8, 20 min; lane 9, 20 μL of binding mixture (40% of sample filtered for each of lanes 1–8); lane 10, 2 μL of control DNA ($^1/_{30}$th of DNA in total reaction mixture).

region into mRNA. The *tet-trp* promoter fragment, however, exhibited a more rapid binding by RNA polymerase.

To compare the relative binding rates of some of the *trp* promoter derivatives, mixed binding experiments on unlabeled fragments were performed. This sort of experiment exposes all promoters in the mixture to the same RNA polymerase conditions, and upon protein binding, elution, and separation by gel electrophoresis, the proportion of each promoter fragment in the mixture can be quantitated (Maquat & Reznikoff, 1978). This experiment was conducted on a mixture of fragments from plasmids containing the *tet-trp* promoter (pDR250) and the *trp* promoter (pDR210), and a binding curve similar to Figure 2 was obtained (data not shown). In these binding experments, the rate of binding of the *bla* promoter could also be monitored as an internal control. With the RNA polymerase concentration used, the binding rate of the *bla* promoter was not significantly different than that found previously (Russell & Bennett, 1981). An experiment of this type comparing two very similar *trp* promoters is shown (Figures 3 and 4). These two promoters differ by only the 13- and 17-bp flanking regions beyond −39 and +2 of the *trp* promoter. In vivo studies (Russell et al., 1984) suggest that expression is higher in pDR210 than in pDR702. To determine if the binding rates of the *trp* promoters of pDR702 and pDR210 are different, a *Bsp*RI digest of the two plasmids was subjected to RNA polymerase binding. Figure 3 shows a photograph of the stained gel of the filter binding experiment. Figure 4 shows the percent of fragment bound for each promoter as calculated from densitometry of band intensities. The curve for the binding of the β-lactamase promoter is not shown but is similar to the curve generated from end-labeled binding experiments and reported previously (Russell & Bennett, 1981). It appears that a change in 30 bp of DNA (17 and 13 bp on either side of the 41-*trp* promoter) may result in a slightly faster rate of in vitro binding but a weaker level of in vivo expression.

The plasmids pDR702-21 and pDR702-23 differ only in the orientation of the dCT/dAG 8-bp insert downstream of the +2 site of the 41-bp *trp* PO (Russell et al., 1984). Both of these *trp* promoters show a slowed rate of polymerase binding when compared to their parental plasmid pDR702 (Table II). These insertion pDR702 *trp* promoter variants increase in vivo galactokinase activity compared to pDR702 (Russell et al., 1984).

Effects of Temperature on Open Complex Formation. Temperature binding curves have been carried out for several of the promoters described above (Figure 5). For example,

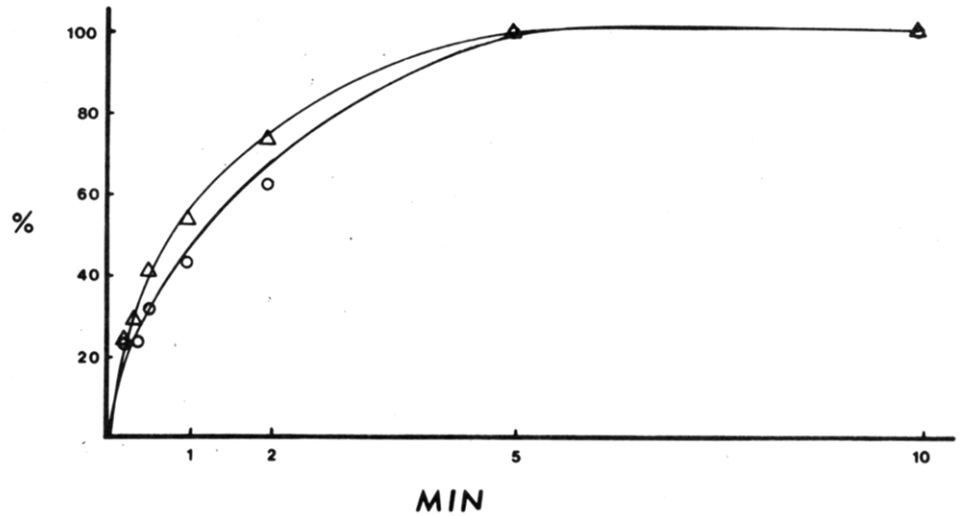

FIGURE 4: Open complex formation of pDR210 and pDR702. The rate of open complex formation is plotted as a percent of filter-bound complexes at 10 min from the gel shown in Figure 3. Triangles indicate *trp* promoter pDR702 binding; circles indicate *trp* promoter pDR210 binding.

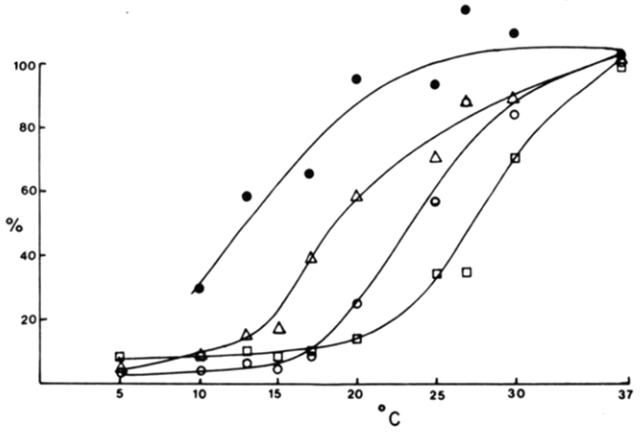

FIGURE 5: Transition temperatures of hybrid promoters. Curves are plotted as the percent of filter-bound complexes at 37 °C. Open circles, *trp-lac* promoter; open triangles, *lac*UV5 promoter; open squares, *tet-lac* promoter; closed circles, anti-*tet* promoter.

the *bla* promoter and the *tet*(19) promoter (a *tet* promoter with a 19-bp spacing between the −35 and −10 regions, presumably a measurement of the anti-*tet* promoter) both have very low transition temperatures (13 °C, see Figure 5). Both promoters also appear to have weak in vivo activity and fast in vitro binding. The *trp-lac* promoter is somewhat higher in transition temperature than *lac*, although it has greater in vivo activity. These temperatures differ from those reported by Kirkegaard et al. (1983), who used an unwinding assay and an abortive initiation assay vs. temperature. The difference could be due to the differences between the detection systems (in their system, no unwinding by the *trp* promoter was observed; however, *trp* promoter binding can be detected by the filter binding assay) or the other conditions of the reaction. Two promoters, *tet-lac* and *trp*, have similar, high transition temperatures and have slower rates of in vitro binding. Several *trp* promoter derivatives were also examined. Although the rate of open complex formation is slowed in the latter two, pDR702, pDR702-21, and pDR702-23 all have similar transition temperatures while the pDR210 derivative binds at a slightly higher transition temperature (Table II). An anomaly was noted in studies of the binding of the *tet-trp* promoter. RNA polymerase appears to bind 30% of the available *tet-trp* promoter fragments at low temperature (2.5–5 °C). The binding level increases gradually to 15 °C and then increases rapidly above 15 °C to form a more usual transition curve. Table II lists this latter transition of about 23 °C, but it must be kept in mind that the binding complex and behavior of this promoter fragment may be different than others.

If the effect of temperature on binding is due to the ease of duplex DNA melting, one might expect a correlation between the AT content of the promoter and its transition temperature. However, when one compares AT content, temperature data, and in vivo expression, there is no clear correlation (data not shown).

There seems to be a general trend between the rate of binding and the transition temperature. The promoters that have the lowest transition temperature are also among the fastest binding in vitro, while those with the higher temperatures of transition tend to bind slower.

Productive Initiation. The rate of productive initiation measures the time it takes for a stable, open complex to form an elongation complex. RNA polymerase and promoter restriction fragments are incubated to form an open complex. An initiation mixture of heparin and all four NTPs (including [α-^{32}P]-CTP) is then added to begin initiation and elongation. Heparin will inactivate any free RNA polymerase at this step.

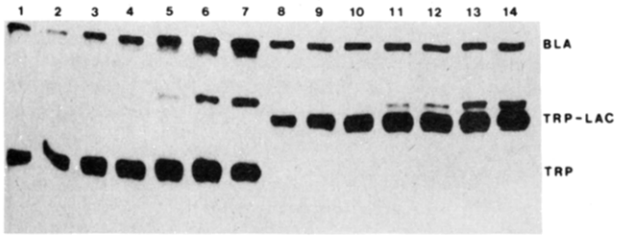

FIGURE 6: Electrophoresis of productive initiation RNA. Autoradiograph of two productive initiation experiments is shown. Lanes 1–7 show experiment using pDR210 (*trp* promoter); lanes 8–14 show experiment using pDR540 (*trp-lac* promoter). The RNAs produced from the *bla*, *trp*, and *trp-lac* promoters are indicated. The other bands are presumably RNAs produced from other promoters on the plasmids used for templates (see text): lanes 1 and 8, 15 s; 2 and 9, 30 s; 3 and 10, 45 s; 4 and 11, 1 min; 5 and 12, 2 min; 6 and 13, 5 min; 7 and 14, 10 min.

At various times, aliquots of the elongation reaction are added to a termination mixture which contains rifampicin and all four NTPs. Rifampicin will prevent any initiation that had not begun at the time of addition but will allow elongating complexes to continue to completion. McClure & Cech (1978) have shown that rifampicin blocks translocation after the first phosphodiester bond is formed. After time to complete elongation is allowed, the reaction is stopped, and the RNA products are analyzed on denaturing gels. By following the time course of RNA production, one can estimate the time required for the open complex to transfer to an elongation complex.

The templates used in these experiments consisted of plasmid DNA that was cleaved with either *Bsp*RI or *Bsp*RI + *Hin*fI. It should be noted that there are four promoters on pKO-1, the vector carrying the cloned promoters to be analyzed [the β-lactamase promoter, a cAMP–CRP-dependent promoter described by Queen & Rosenberg (1981), and two promoters in the origin of replication (Selzer et al., 1983)]. Therefore, an assay of pDR210, for example, would include the *trp* promoter and four others. The *bla* promoter transcript was used in analyzing the RNA products as an internal standard to ensure that each set of reactions was comparable.

Productive initiation measurements have been reported for the *lac*UV5 promoters, $t_{50\%}$ = 60 s (Stefano & Gralla, 1979; Munson & Reznikoff, 1981), and Horowitz and Platt have observed that *trp* productive initiation is more rapid than *lac*UV5. Table II and Figures 6 and 7 indicate that *trp*, *tet-trp*, and *bla* all have very rapid rates of productive initiation while *trp–lac* appears to be near to the rate reported for *lac*UV5. Results also suggest the rate for the *tet-lac* promoter is similar to that for *lac*UV5.

It has been proposed that the rate of productive initiation may depend in part upon the mRNA sequence. Carpousis & Gralla (1980) have shown that productive initiation can be altered by limiting the nucleotide concentration of nucleotides incorporated into the first two residues of the mRNA. Munson & Reznikoff (1981), however, observed no effect on varying the concentration of the fourth residue from 10 to 200 μM. In all of the experiments described here, CTP was used as the labeled nucleotide (Figure 8). For the promoters with the *lac*UV5 front half, the first CTP is incorporated at the ninth position, while for *trp* (pDR210) and *tet-trp* (pDR250) the first CTP is at the sixth position. However, for pDR702, the first CTP is incorporated at +3, while pDR702-21 has a region of alternating CT starting at +3. Therefore, pDR702-21 would be the most likely candidate to show sequence-specific effects upon the rate of productive initiation at several concentrations of CTP. Figure 8 shows the effects of increasing

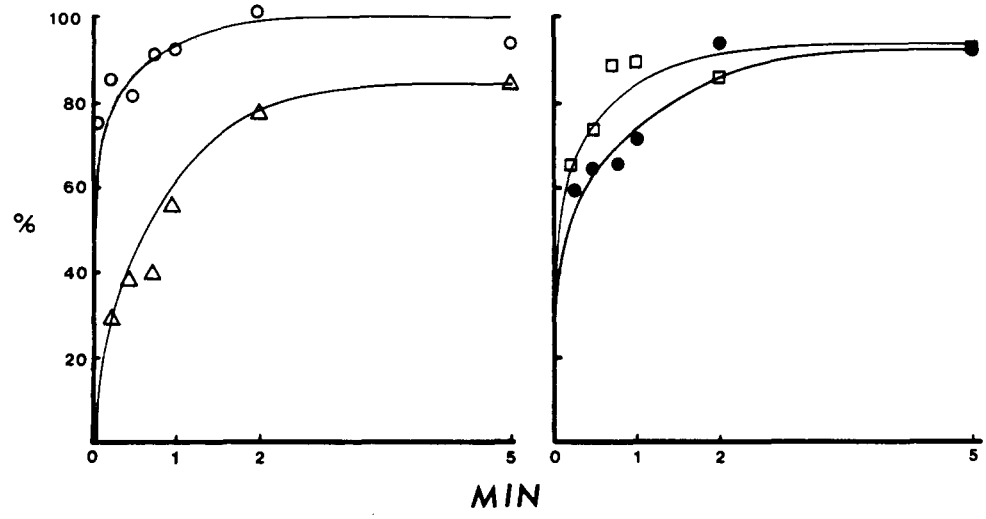

FIGURE 7: Productive initiation of selected promoters. Curves are the mean of multiple determinations plotted as a percent of RNA produced at 10 min. Circles, *trp* promoter pDR210; triangles, *trp-lac* promoter pDR540; squares, *tet-trp* promoter pDR250; closed circles, *bla* promoter (mean from each of above experiments).

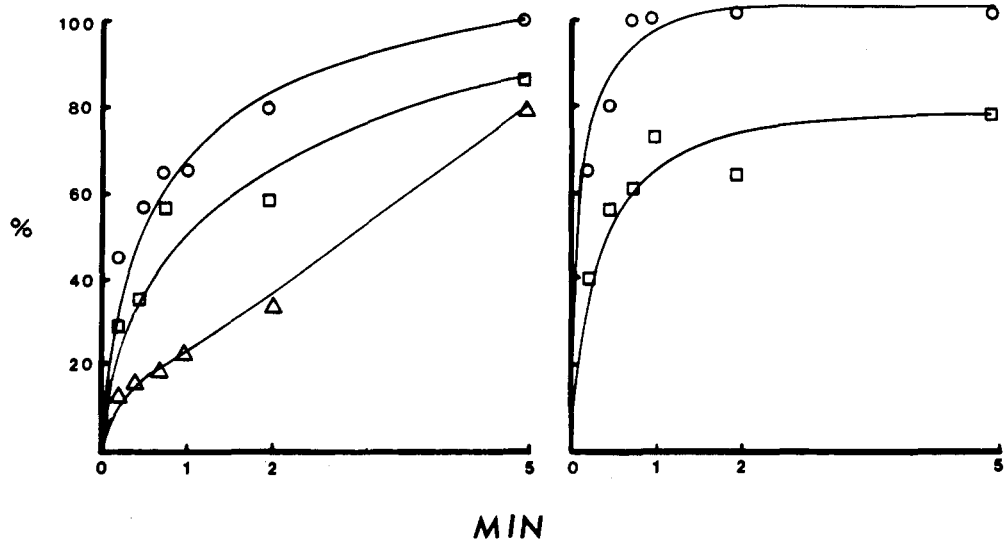

FIGURE 8: Effect of nucleotide concentration on productive initiation. Curves are plotted as the percent of RNA produced at 10 min. Conditions are as described under Experimental Procedures except the CTP concentration has been altered in each experiment. The left panel is the productive initiation of pDR702-21 at (circles) 50, (squares) 20, or (triangles) 5 μM CTP. The right panel is the productive initiation of pDR702 at (circles) 20 or (squares) 5 μM CTP.

the CTP concentration from 5 to 50 μM for three related promoters. The most striking changes are observed with pDR702-21, which shows a significant increase in the rate of productive initiation as CTP concentration is increased from 5 to 50 μM. The results from pDR702-21 compared with the other two related promoters clearly show the sequence in the transcribed region can affect the rate of productive initiation, if low concentrations of nucleoside triphosphates are used.

A comparison of all the promoters shown in Table II does not show an observed correlation between productive initiation and either the rate of open complex formation or the in vivo promoter expression.

Discussion

These experiments were carried out to determine if in vitro measurements can be correlated with the in vivo promoter expression studies described earlier (Russell & Bennett, 1982b) and to assess whether specific regions of promoter sequence can alter specific aspects of the promoter–RNA polymerase interaction. Several previous reports have suggested that the rate of in vitro open complex formation correlates with relative in vivo expression. For example, Maquat & Reznikoff (1978) measured the rate of open complex formation of various *lac* promoter mutants by filter binding and concluded there was a direct relationship between the rate of open complex formation and in vivo promoter activity (*lac* promoter mutants with stronger in vivo activity also bound polymerase faster and to a more complete degree in vitro). Oppenheim & Yanofsky (1980) compared four *trp* promoter mutants and observed the slowest in vitro rate coincided with the weakest in vivo mutants while the fastest in vitro rate coincided with the strongest in vivo promoter. However, they also showed that a mutant half as strong in vivo as the wild-type *trp* promoter bound at the same in vitro rate as the wild type, A series of papers by Stefano & Gralla (1979, 1980, 1982a,b) studied the rate of in vitro open complex formation among a group of *lac* promoter mutants. These data indicated the level of in vivo expression paralleled the rate of open complex formation in vitro.

In the above studies of the *lac* system, only small sequence changes in the *lac* promoter were being studied, and in most cases, the transcribed regions were identical. The experiments described here have sought to compare a series of different promoters, and while a standard gene expression system was

used to minimize nonpromoter effects on expression, factors other than promoter differences can lead to changes in in vivo expression. Differences in plasmid copy number and stability can affect the level of expression observed; however, those promoter plasmids examined (Russell et al., 1984) have not revealed large effects of this kind. Since the mRNA from different promoters will have different 5′ ends, they may differ in their mRNA stability or translational efficiency which will affect the measurement of protein levels. Also, the presence of operators regulating expression from the *lac* and *trp* promoters requires complete induction in vivo to enable evaluation of the full activity of these promoters. The induction conditions used were sufficient to completely relieve repression of the *tet-trp* promoter vs. the homologous repressor minus strain (E. A. Auger, unpublished experiments). However, the possibility for incomplete attainment of steady-state conditions for some strong promoters might exist which would thus underestimate the relative strength of these promoters. Therefore, while individual values for in vivo promoter activity (Table I) may not be exact measures of their promoter strength, the relative order of the promoters is supported by reports using other systems. The strongest promoter seems to be the *trp-lac* hybrid which has been reported to express approximately 5–10 times that of *lac*UV5 (de Boer et al., 1982, 1983; Amann et al., 1983). The *trp* promoter has been reported to be approximately 2 times as active as the *lac*UV5 promoter (Edge et al., 1983; Shirakawa et al., 1984). Studies on the *bla* promoter indicate it is weaker than *lac*UV5 (Harris, 1983) and much weaker than *trp* (Edman et al., 1983), and its activity is near that of the anti-*tet* promoter (von Gabain et al., 1983).

The data summarized in Table II suggest the relationship of in vitro binding rate to in vivo activity may not hold when less related promoters are compared. For example, the fastest binding promoters, *bla* and anti-*tet*, both show rather weak in vivo expression. The slowest binding promoters, *trp* (pDR210, pDR702-21, pDR702-23), also show strong in vivo expression. This relative order has been found in isolated fragment binding experiments as well as mixed binding experiments. Of the *lac*UV5 derivatives studied, the least active in vivo is also the slowest binding.

It has been speculated that the transition temperature measured in vitro may reflect a form of regulation in vivo that involves the melting of duplex DNA during open complex formation. For example, Horn & Wells (1981) suggested that promoters that melt at lower temperatures may be more stable and efficient promoters. The data summarized in Table II do not suggest a correlation between the in vitro transition temperature and promoter efficiency as measured by in vivo gene expression. In fact, promoters in this study that have the lowest transition temperatures are also the weakest in vivo promoters. While the transition temperature does not appear to be related to in vivo activity, it does appear to roughly correlate with the rates of in vitro open complex formation. The fastest binding promoters also bind at lower temperatures while slower binding promoters generally also require higher temperatures to reach 50% of binding. It could be argued that the slower rate of open complex formation for promoters such as *trp* may be due to the higher energy required to melt the duplex DNA during complex formation. However, Kadesch et al. (1982) have shown that the melting reaction is not the rate-limiting step of open complex formation for the T7-A1 promoter. This observation also may be relevant to the parameters observed for pDR702-21 and pDR702-23.

The productive initiation data are summarized in Figures 6–8 and Table II. Carpousis et al. (1982) observed an inverse relationship between the rate of open complex formation and productive initiation for several closely related *lac* promoter mutants. This relationship cannot be generally extended to the group of promoters studied here. However, only the promoters containing the *lac*UV5 front half had rates slow enough to measure by using this assay. Most of the promoters studied by other workers have very fast rates of productive initiation [see, for example Horn & Wells, (1981) and Nierman & Chamberlin (1980)].

One interesting feature of productive initiation is apparent from these data. Table II shows the sequence on the transcribed region can significantly affect the half-time of productive initiation under conditions of limiting nucleotide concentration. This was not observed for the *lac*UV5 promoter when the concentrations of the third and fourth residues of the RNA were varied from 10 to 200 μM (Munson & Reznikoff, 1981). It is not known if the differences observed are due to the nature of the transcribed sequences or to intrinsic differences in the *lac*UV5 and *trp* promoters.

These data also suggest that both the −35 and −10 regions can independently affect a particular aspect of promoter function. For example, compare *trp* and *trp-lac* promoters. The former binds much slower than the latter, presumably due to differences between *trp* and *lac* front halves. Comparison of *trp-lac* (fast) and *tet-lac* (slower), on the other hand, shows that changing the back half also can affect the rate of open complex formation. Stefano et al. (1980) have observed that mutations in either the −10 or the −35 region can independently affect the in vitro rate. In addition, it appears that changing the flanking regions near the *trp* promoter may alter the rate of open complex formation. It appears that altering the flanking region near +2 can alter the in vitro rate of binding as well as in vivo expression.

One in vitro promoter characteristic not measured here can also be discussed. The stability of promoter complexes has been measured by following the rate the open complex dissociates. The dissociation for the *bla* promoter is $t_{50\%} \sim 6$ h (Russell & Bennett, 1981). This measurement has also been reported for several other promoters including *trp* ($t_{50\%} \sim 1$ h; Oppenheim & Yanofsky, 1980) and four *lac* promoter mutants ($t_{50\%} \sim 2$–6 h depending upon the mutant). Among these promoters, those that bind quickly tend to dissociate more slowly. Considering three promoters examined here, *bla*, *lac*UV5, and *trp*, it would appear that promoters which form the more stable complexes are weaker in vivo promoters. This relationship cannot be extended to all the promoters considered here. As more data become available on the stability of promoter complexes, this aspect may be further clarified.

The in vitro rate of open complex formation has previously been used to show a relationship to in vivo expression among groups of closely related promoter mutants. A more general compilation has been published which shows a general correlation between $K_B K_2$ vs. promoter sequence homology (Mulligan et al., 1984). It may be that different promoters may represent different classes of promoter binding and expression. Within the same class of promoters (for example, the *lac* promoter derivatives), a correlation between in vivo activity and in vitro binding rates may be clear. However, comparison across different classes may be more difficult to establish.

It should be noted that the above experiments and all of the in vitro characteristics described here are measured by using linear DNA fragments. In vivo promoter expression is from negatively supercoiled DNA and occurs in the presence of a variety of DNA binding proteins and RNA polymerase

modifying proteins. These factors which are not present in any of the in vitro assays may play an important role on certain promoters during in vivo transcription and expression.

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