Parameters Affecting the Elongation Block by RNA Polymerase II at the SV40 Attenuator 1 in Vitro[†]

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ABSTRACT: We have previously suggested that folding of the SV40 attenuator RNA 1 into two hairpin elements leads to a block of transcription elongation. Using site-directed mutagenesis, we created three templates that either strengthened or weakened the proposed hairpin structures. The mutated and wild-type templates were cloned downstream from the adenovirus 2 MLP, and transcription patterns were compared among the templates, in cell-free extracts. In this report, we show that at the standard temperature (30 °C) the elongation block occurs at elevated KCl concentrations (0.5–1.2 M KCl) while at high temperature (65 °C), although the transcription elongation rate increases, the block to elongation occurs at lower KCl concentrations (<0.5 M KCl) as well. Since under the conditions tested the extent of the elongation block is directly dependent on the stability of the hairpin structure of the RNA, as assessed by a comparison of transcription of the wild-type and mutated templates, we suggest that elevated KCl concentrations and high temperatures are favored conditions for optimizing the processes whereby the hairpin structures are recognized by the polymerase as an attenuation signal. Moreover, the present experiments indicate that cellular elongation factors are not directly involved in modulating the extent of the elongation block at the SV40 attenuator 1 in vitro. The SV40 attenuator 1 is the only eukaryotic system known to date which has been shown to have structural characteristics so similar to ρ-independent terminator in prokaryotes. We discuss the similarities between the mechanisms which lead to elongation blocks at these eukaryotic and prokaryotic sites.

In SV40, the attenuator RNA 1 originates from the major late initiation site at nucleotide 325 and terminates about 95 nucleotides downstream at nucleotides 419–421. Attenuator RNA 2, which also initiates at the major late initiation site, terminates 150 nucleotides downstream at nucleotide 475, and a 21 base pair DNA fragment was found to direct transcription attenuation at this site (Kessler et al., 1991). The attenuator RNA 1 was identified following in vitro elongation of nascent RNA in several systems including viral transcription complexes, SV40 minichromosomes, and isolated nuclei (Hay et al., 1982; Skolnik-David et al., 1982; Pfeiffer et al., 1983; Aloni & Hay, 1985). Examination of the sequence of the attenuator RNA 1 showed that the 3′-end is U-rich and is preceded by self-complementary sequences enabling the formation of two stem and loop structures (see Figure 1A). Because the second stem and loop structure is not highly stable (−12 kcal), we postulated that it is part of a more complex structure that includes the first stem and loop structure (−23 kcal) and that together they lead to the elongation block. We further suggested that the SV40 attenuator RNA 1, by folding into an alternative secondary structure that is not followed immediately by a stretch of U residues (see Figure 1B), enables transcriptional readthrough of this site (Hay et al., 1982) and that these structures together function as a regulated block to transcription elongation (Hay et al., 1982; Skolnik-David & Aloni, 1983; Aloni & Hay, 1985). In this figure, one secondary structure, designated readthrough (see Figure 1B),

would eliminate the transcription elongation block. The second set of secondary structures, designated attenuation (see Figure 1A), would lead to a block to transcription elongation at this site and potentially to termination. Evidence that RNA secondary structure is indeed involved in the block to elongation at the SV40 attenuation site was provided by the observation that the incorporation of IMP instead of GMP, which leads to destabilization of the RNA secondary structure, alleviated the elongation block at the SV40 attenuation site (Hay & Aloni, 1984). In more recent studies using viral transcription complexes (Kessler & Aloni, 1989), or the late promoter-proximal region of SV40 and cell-free transcription, we have shown that the secondary structure of the RNA is indeed an integral part of the attenuation mechanism (Resnekov et al., 1989). Moreover, transcription complexes recognize the RNA secondary structures within the leader region of the late SV40 message and pause or terminate transcription even when they are placed downstream of the MLP of Ad2 (Resnekov et al., 1989). In the latter studies, only in the presence of Sarkosyl and when the elongation reaction was carried out at 45 °C were attenuated transcripts synthesized, indicating an active elongation block. On the basis of these studies, we could not distinguish between the possibilities that this Sarkosyl concentration and elevated temperature either inactivate cellular elongation factors, which prevent the elongation block, or optimize the processes involved in the attenuation mechanism.

In order to distinguish between these two possibilities, we have recently developed a promoter-dependent assay where initiation of transcription and brief elongation take place in crude cellular extracts. Elongation complexes are then partially purified using gel filtration chromatography at high KCl concentrations. The column-purified transcription complexes, which are depleted of elongation factors, can serve as a model system for determining the activities of factors during
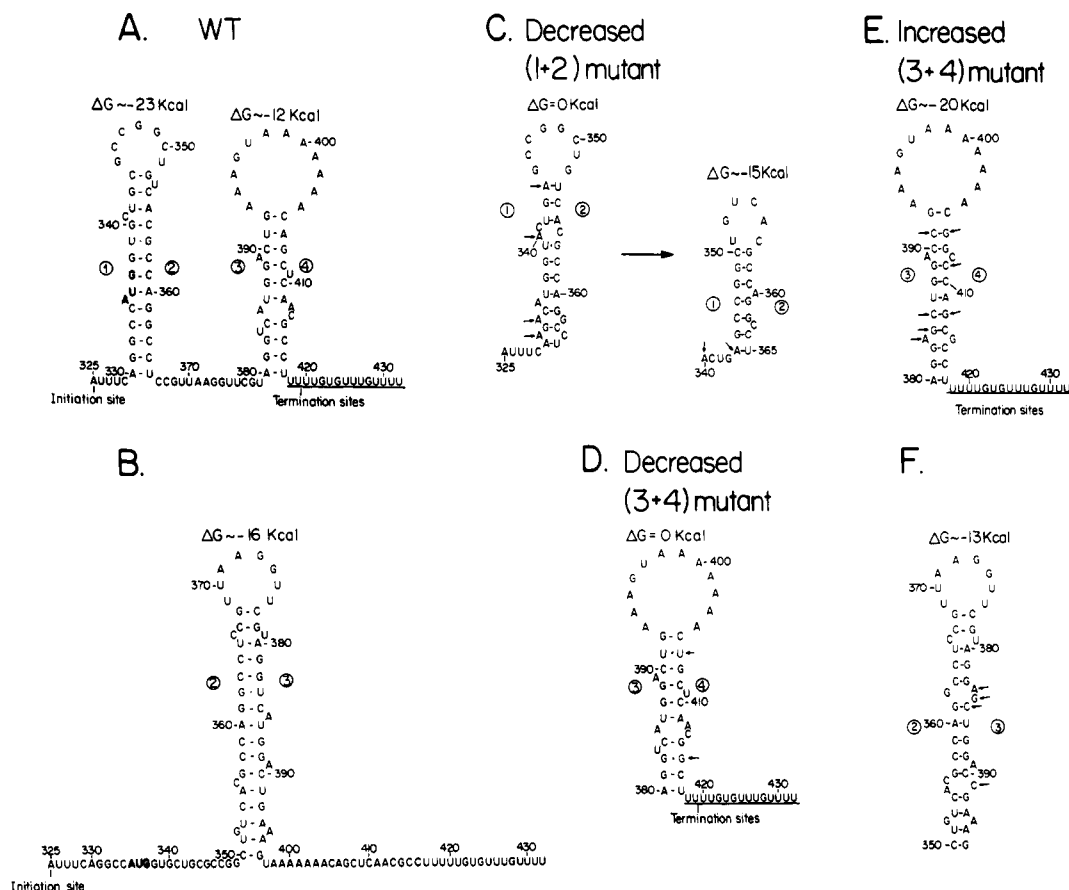
FIGURE 1: Potential secondary structures in the attenuator RNA of WT SV40 and three mutants. Arrows show point mutations. The circled numbers refer to individual sides of the stem and loop structures. (Panel A) The potential stem and loop structures followed by a U-rich region that are postulated to lead to a block of transcription elongation and which together are designated the "attenuation conformation" (Hay et al., 1982). (Panel B) The alternative stem and loop structure of the same RNA which is postulated to lead to transcription readthrough and which is designated the "readthrough conformation". Note that stem (2+3) is not followed by a stretch of U's. (Panel C) The effect of the mutations of the decreased (1+2) mutant on the (1+2) stem and loop structure. An alternative (1+2) secondary structure that can be formed as a result of the mutations is also shown and is less stable than the WT (1+2) structure. (Panel D) The effect of the mutations of the decreased (3+4) mutant on the (3+4) stem and loop structure. (Panel E) The effect of the mutations of the increased (3+4) mutant on the (3+4) stem and loop structure. (Panel F) The effect of the mutations of the increased (3+4) mutant on the (2+3) stem and loop structure.

elongation (Bengal et al., 1989). Using this approach, we have recently shown that the block to elongation at the Ad2 attenuator (Bengal et al., 1989, 1991), SV40 attenuator 2 (Kessler et al., 1991), and MVM attenuator (Resnekov & Aloni, 1989; Krauskopf et al., 1991) is modulated, at least in part, by the cellular elongation factors.

In the present study, we show that elevated KCl concentration and high temperature are favored conditions for eliciting the in vitro elongation block at the SV40 attenuator 1. By comparison of transcription of wild-type and mutated templates, we show that under the conditions tested the extent of the elongation block is directly dependent on the stability of the hairpin structures of the RNA. We therefore suggest that elevated KCl concentrations and high temperatures optimize the processes whereby the attenuator RNA secondary structures and the stretch of U residues lead to transcription attenuation. Moreover, in contrast to the control at the Ad2 attenuator (Bengal et al., 1991), SV40 attenuator 2 (Kessler et al., 1991), and MVM attenuator (Kruaskopf et al., 1991), the present experiments show that cellular elongation factors are not directly involved in modulating the extent of the elongation block at the SV40 attenuator 1 in vitro.

MATERIALS AND METHODS

Chemicals. Nucleoside triphosphates and creatine phosphate were purchased from Sigma Chemical Co. [α-^{32}P]UTP
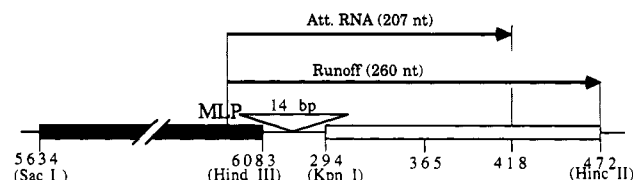
FIGURE 2: Schematic drawing of the constructs and the RNA lengths. The black arrows show the length, in nucleotides, of the runoff and attenuator RNAs. The functional promoter of this plasmid is the MLP of Ad2 (black box). This is followed by the *KpnI–MboI* fragment of SV40 (open box). The triangle represents a 14 base pair linker inserted in the polylinker between the Ad2 MLP and the SV40 sequences. For the experiments described, the templates were digested with *HincII*.

and [α-^{32}P]CTP (400 Ci/mmol) were purchased from Amersham Corp., and Sephacryl S-1000 was from Pharmacia.

Constructs and Mutations. The mutations indicated in Figure 1 were introduced by site-directed mutagenesis, as described (Resnekov et al., 1989). The SV40-mutated or wild-type sequences were then cloned into pGEM MLP containing the Ad2 MLP fragment as described (Resnekov et al., 1989). The extended construct contains an additional 14 base pairs *SmaI* adaptor at the *SmaI* site of the polylinker (see Figure 2).

In Vitro Transcription with HeLa Whole Cell Extract (WCE). WCE was prepared as described (Manley et al., 1980). Ten microliters of WCE was incubated for 20 min at

30 °C with ~1 μg of DNA and 4 mM creatine phosphate in a final volume of 15 μL (preinitiation step). ATP, GTP, and CTP (final concentrations of 500 μM) and 30 μCi of [α-^{32}P]UTP in 5 μL were added and incubated for a pulse of 35 s at 30 °C (initiation step). The reaction was then diluted with 280 μL of "elongation chase buffer" (20 mM Tris-HCl, pH 7.9, 5 mM MgCl$_2$, 0.1 mM EDTA, 1 mM DTT, 4 mM creatine phosphate, and 500 μM each of the four ribo-NTP's). KCl at the indicated concentrations was also included in the chase reaction. The reaction was allowed to proceed for 20 min (elongation step). The elongation step reaction was stopped by the addition of 20 mM EDTA, 0.4% SDS, 200 μg/mL proteinase K, and 10 μg of tRNA and incubated at 30 °C for 15 min. RNA was phenol/chloroform-extracted, purified by centrifugation through a Sephadex G-25 minicolumn, and collected by ethanol precipitation and centrifugation.

Partial Purification of Transcription Complexes by Sephacryl S-1000 Chromatography. The preinitiation step as described above was employed, but at 8 times the volume; 500 μM ATP and 240 μCi each of [α-^{32}P]CTP and [α-^{32}P]-UTP were then added for 2 min at 30 °C and then applied directly onto a Sephacryl S-1000 minicolumn (4-mL volume) that had been equilibrated with 0.8 M KCl, 20 mM Tris-HCl, pH 7.9, 5 mM MgCl$_2$, 0.1 mM EDTA, 10% glycerol, and 1 mM DTT. The first four fractions containing the radioactive material were collected [see Bengal et al. (1991)]. Samples from the pooled fractions were diluted with elongation chase buffer, bringing the KCl to the concentrations as indicated in the figures. In vitro transcription reactions were then allowed to proceed for an additional 30 min at 30 °C, and RNA was extracted as described above.

Polyacrylamide Gel Electrophoresis (PAGE). RNA was resuspended in 10 μL of 90% formamide and analyzed on 6% polyacrylamide gels [bis(acrylamide): acrylamide ratio of 1:19] containing 7 M urea (Schwarz/Mann) and 1× TBE (89 mM Tris–borate, pH 8.3, 89 mM boric acid, and 2 mM EDTA). Electrophoresis was carried out at a constant current of 20 mA.

RESULTS

Possible Effects of Mutations in the Putative Secondary Structures of the SV40 Attenuator RNA 1 on the Elongation Block in Vitro. Three sets of mutations were created by site-directed mutagenesis in the SV40 DNA encoding the attenuator RNA 1. The first set of mutations completely destabilized the (1+2) stem and loop as is diagrammed in Figure 1C (0 kcal); however, a weaker alternative structure (−15 kcal) can form as the result of the mutations (Figure 1C). Since all the mutations were introduced in stem 1, the stability of the (2+3) structure was not affected by them, enabling us to determine if the (1+2) and (3+4) structures are part of a complex structure in which formation of (1+2) allows for and enhances the formation of (3+4). If the (1+2) and (3+4) structures function independently of each other to lead to the block to elongation, then this construct would not be expected to significantly affect the accumulation of the (3+4) RNA, since it does not affect the stability of the (3+4) structure or the stability of the (2+3) readthrough structure. However, if the (1+2) and (3+4) stems and loops are parts of the same structure, then the accumulation of the attenuator RNA may be affected significantly. The second set of mutations decreases the stability of the (3+4) stem and loop and is diagrammed in Figure 1D. Since the mutations were introduced into stem 4, this construct is expected to increase

the stability of the (2+3) stem and loop and hence to decrease the accumulation of the attenuator RNA. The purpose of the third set of mutations was to stabilize the (3+4) stem and loop structure and is diagrammed in Figure 1E. This contruct is expected to lead to increased accumulation of the attenuator RNA due to the increased stability of the (3+4) stem and decreased stability of the (2+3) readthrough stem (compare Figure 1B and Figure 1F).

The WT and three mutated constructs were cloned downstream of the Ad2 MLP (Figure 2). This promoter was chosen as it is a strong promoter, and it has been well characterized in cell-free systems. Moreover, in order to establish that the attenuator RNA and runoff initiate at the Ad2 MLP and terminate at a distance from it as measured by their migration on the gel, a 14 base pair *SmaI* linker was inserted between the Ad2 MLP and the SV40 sequences (see Figure 2), and the length of the transcripts was compared with those of the regular constructs in all experiments described below.

All plasmids were transcribed in vitro using HeLa WCE under pulse–chase conditions. Thus, following a pulse with [α-^{32}P]UTP for 35 s the reactions were diluted 20-fold with buffer containing various KCl concentrations. Under these conditions, the nascent RNAs were shorter than 50 nt at the time of the chase (data not shown). By carrying out transcription under these conditions, it is possible to study single rounds of transcription elongation and termination and vary conditions over a wide range with no possibility of effects due to alteration of initiation.

Alterations of KCl Concentrations during Elongation Affect the Synthesis of SV40 Attenuator RNA 1. Elevated concentrations of monovalent cation have been shown to increase the termination efficiency of *Escherichia coli* RNA polymerase at some but not all termination sites (Neff & Chamberlin, 1980; Reynolds et al., 1992) and to increase the recognition of termination sites by purified mammalian RNA polymerase II (Dedrick et al., 1987). We have recently shown that exposure of briefly initiated transcription complexes to elevated KCl concentrations results in the dissociation of general elongation factors from the complexes, resulting in a block to elongation at specific attenuation sites (Bengal et al., 1989, 1991). Since elevated KCl concentrations may affect protein–nucleic acid binding as well as the stability of the RNA secondary structures which are an integral element of the attenuation signal in SV40 (Hay et al., 1982; Hay & Aloni, 1984; Aloni & Hay 1985; Resnekov et al., 1989; Kessler & Aloni, 1989), we evaluated the effect of elevated KCl concentrations on transcription through the attenuator in wild-type (WT) template and templates encoding for decreased (1+2), decreased (3+4), and increased (3+4) structures. Transcription was carried out in HeLa WCE under pulse–chase conditions, and the KCl concentrations were varied during the elongation step. As can be seen in Figure 3, transcription of all four plasmids led to the accumulation of two transcripts: a runoff transcript and a shorter transcript of about 207 nt that we termed attenuator RNA (see scheme in Figure 2). This corresponds to an RNA with a 3′-end near nucleotide 419 (SV40 numbering). Both transcripts were found to be sensitive to 0.5 μM α-amanitin, indicating that they are transcribed by RNA polymerase II (data not shown). Moreover, in all plasmids, the synthesis of the attenuator RNA was dependent on the KCl concentration during elongation. The attenuator RNA is recognizable when the KCl concentration is above 0.5 M KCl, except with the increased (3+4) plasmid where it is already recognized with 0.3 M KCl. It is also apparent that with increasing concentrations of KCl there
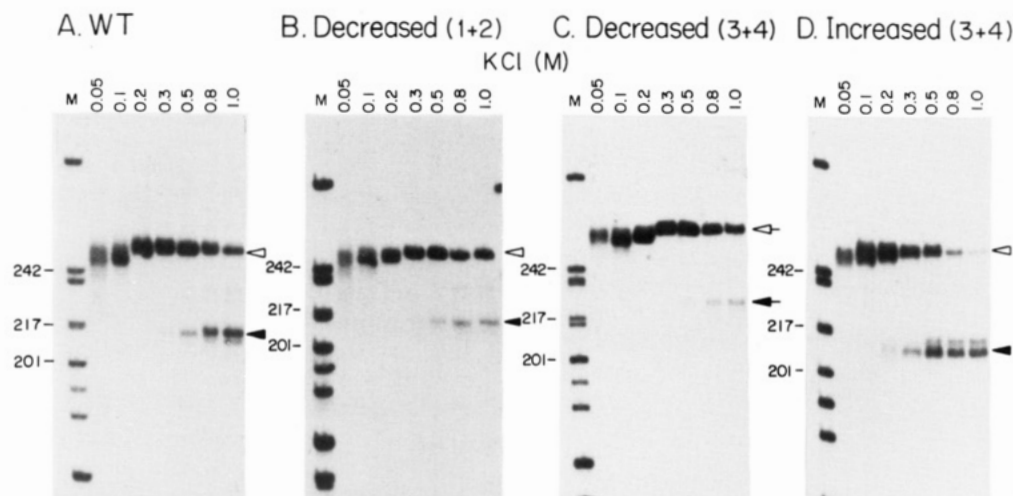
FIGURE 3: Size analysis of the RNAs produced in WCE from four templates under different KCl concentrations during transcription elongation. The different constructs are described in Figure 1. A schematic representation of the templates digested with *Hinc*II is shown in Figure 2. The runoff (open arrowhead) and the attenuator RNAs (solid arrowhead) are indicated. The arrows (in panel C) indicate the shift in length of the runoff and attenuator RNAs produced on the "decreased (3+4)" construct, in which 14 bp was inserted downstream from the transcription initiation site in the polylinker between the Ad2 MLP and the SV40 sequence (see Figure 2 for schematic diagram of the constructs). Transcription reaction components were preincubated at 30 °C for 20 min without nucleotides. Nucleotide triphosphates were then added to a final concentration of 500 μM (ATP, CTP, GTP) in the presence of 20 μCi of UTP (400 Ci/mmol) at 30 °C for 35 s (pulse labeling). The reaction was then diluted to 300 μL with elongation chase buffer, as described under Materials and Methods, containing the indicated concentrations of KCl. Transcription was continued at 30 °C for 20 min as described under Materials and Methods. Lane M shows size marker (in nucleotides) of pBR322 digested with *Hpa*II and end-labeled. The same markers were used in all figures.

is a gradual increase in the synthesis of the attenuator RNA and a corresponding decrease in the synthesis of the runoff RNA. Optimal total incorporation (attenuator RNA plus runoff RNA) is noted when the KCl concentration during elongation is between 0.2 and 0.8 M KCl. Finally, in comparison to the WT plasmid, the synthesis of the attenuator RNA is decreased in the decreased (1+2) and (3+4) mutants and is increased in the increased (3+4) mutant. Note that in the decreased (3+4) mutant there is a 14-nt shift in the lengths of the attenuator and runoff RNAs, corresponding to the insert of 14 bp *Sma*I linker between the Ad2 MLP and the SV40 sequences (see Figure 2), indicating that both RNAs in all plasmids are synthesized from the Ad2 MLP (Resnekov et al., 1989).

A more direct comparison of transcription of the four plasmids is shown in Figure 4, where synthesis of the attenuator RNA in 0.8, 1.0, and 1.2 M KCl is compared. Overall, the increased (3+4) mutant accumulated the most attenuator RNA (>90%) followed by the Wt (≈50%) and the decreased (1+2) and (3+4) mutants (≈25%). The most pronounced differences between the various plasmids were observed in the reactions containing 1.0 M KCl. The data are consistent with the postulated secondary structure with the designated mutations and the model that the (1+2) stem and loop structure is an integral element of the attenuation conformation (see Figure 1A) (Hay et al., 1982; Hay & Aloni, 1984; Aloni & Hay 1985; Resnekov et al., 1989; Kessler & Aloni, 1989). It is worth emphasizing that in the decreased (1+2) plasmid the mutations in strand 1 are located about 80 nucleotides upstream of the site of the elongation block. The appearance in some cases of two attenuator RNA bands supports our previous conclusion that attenuation is a "wobbly" process (Pfeiffer et al., 1983; Resnekov et al., 1989). We conclude that there is a fine balance between the innate RNA structures and environmental conditions.

Elevated Temperatures during Elongation Enhance the Synthesis of SV40 Attenuator RNA 1. Rho-independent termination by *E. coli* RNA polymerase is believed to be a two-step process in which the terminator RNA hairpin first
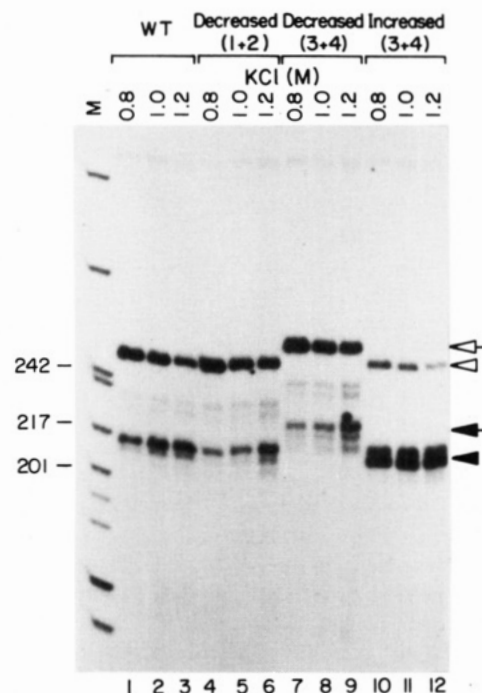
FIGURE 4: Effect of the mutations on the efficiency of attenuation. Preincubation, transcription initiation, and elongation under chase conditions in the indicated KCl concentrations were done as described in Figure 3 and under Materials and Methods. The runoff (open arrowhead) and the attenuator RNAs (solid arrowhead) are indicated. Open and solid arrows indicate the runoff and the attenuator RNAs produced on the decreased (3+4) construct, in which 14 bp was inserted downstream from the transcription initiation site, as shown in Figure 2.

induces the RNA polymerase to pause during elongation (Rosenberg & Court, 1979; Farnham & Platt, 1980). This is followed by release of the RNA from the paused complex due to the instability of a DNA–RNA hybrid in the 3'-tail region and re-formation of the DNA duplex [Farnham & Platt, 1980; Martin & Tinoco, 1980; for a review, see Platt (1981, 1986) and Yager and von Hippel (1987)]. One
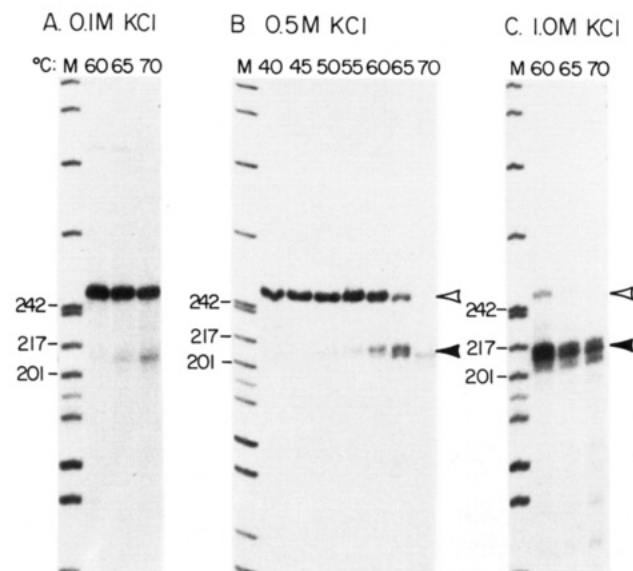
FIGURE 5: Effect of elevated temperature, in various KCl concentrations, on the efficiency of attenuation in the wild-type construct. Preincubation and transcription initiation was performed as described in Figure 3. Elongation was performed in 0.1 M KCl (panel A), 0.5 M KCl (panel B), and 1.0 M KCl (panel C) at the temperatures indicated. The runoff (open arrowhead) and the attenuator RNAs (solid arrowhead) are indicated.

indication as to the role of the rU–dA interaction in the termination process is that melting of the rU–dA hydrogen bonds, by increasing the temperature during elongation, enhances termination [for reviews, see Platt (1981, 1986) and von Hippel et al. (1984)]. We have previously suggested that the SV40 attenuator 1 shares similarities with the prokaryotic ρ-independent terminator (Hay et al., 1982; Hay & Aloni, 1984; Aloni & Hay, 1985), and therefore examined the effect of elevated temperature on transcription through the SV40 attenuator 1 site.

The effect of increased temperature on attenuation was first tested with the WT plasmid using three KCl concentrations: 0.1, 0.5, and 1.0 M. Figure 5 shows that at all KCl concentrations tested, attenuator RNA was observed when the temperature during elongation was above 60 °C. Furthermore, as was observed in Figures 3 and 4, there was a dramatic effect on attenuation when the KCl concentration was above 0.8 M (Figure 5, compare panel C with panels A and B). By comparison of the results in Figures 3 and 5, it is also apparent that there is an additive effect of increased KCl concentration and temperature on attenuation.

In Figure 3, attenuated transcripts are observed when the KCl concentration during elongation is above 0.5 M. In Figure 5, the maximal effect of temperature on inducing the synthesis of the attenuator RNA is noted at 65 °C. We therefore compared the quantities of attenuated transcripts which accumulated with WT and mutated constructs when the elongation reaction was carried out in 0.5 M KCl at 65 °C. Figure 6 shows that with transcription of the increased (3+4) mutant template the greatest amount of attenuator RNA accumulated (≈100%), followed by the WT and the decreased (3+4) mutant (≈50%) and the decreased (1+2) mutant (≈25%) templates. Surprisingly, the quantity of attenuated transcripts was not reduced with the decreased (3+4) mutant template as compared to the WT template. This indicates that [KCl] and temperature may affect different elements or protein–nucleic acid interactions involved in the attenuation process.
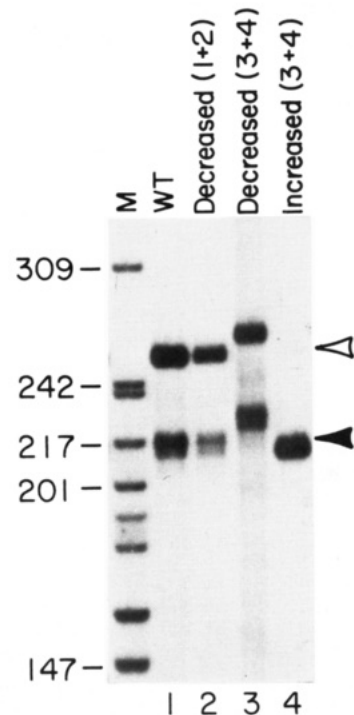

FIGURE 6: Size analysis of the RNA produced from the WT and the three mutated constructs under high KCl concentrations (0.5 M) at elevated temperature (65 °C) during transcription elongation. Preincubation, transcription initiation, and chase reaction were performed as described under Materials and Methods. The runoff (open arrowhead) and the attenuated (3+4) RNAs (solid arrowhead) are indicated. The extended RNAs [decreased (3+4)] have a 14 bp linker inserted downstream from the transcription initiation site as schematically diagrammed in Figure 2.

Rate of RNA Chain Elongation Is Increased at Elevated Temperatures. The previous results show that elevated temperatures at the time of elongation enhance the extent of attenuation. This could be due to impairment of the elongation reaction as a result of elevated temperature and not due to a specific effect of the elevated temperature on the attenuation mechanism. We therefore carried out kinetic experiments in various KCl concentrations and at low and high temperatures during elongation to measure the effects of [KCl] and temperature on the rate of transcription. Figure 7 shows that at all KCl concentrations tested, including 0.05 M KCl (data not shown), increasing the temperature during elongation from 30 to 65 °C dramatically increased the elongation rate. For example, at 30 °C, the 260-nt runoff transcript was synthesized between 60 and 300 s whereas at 65 °C its synthesis required 20 s only, indicating that the elongation rate was increased by 4–15-fold. Elevating the temperature further to 70 °C impaired the elongation reaction, specifically at high KCl concentrations (compare panels B and C). Furthermore, in agreement with the results of Figure 5, elevating the temperature during elongation enhanced the synthesis of the attenuator RNA (indicated by the solid arrowheads in Figures 5 and 7). Thus, the enhancement of attenuation was not due to simple reduction in the rate of RNA chain elongation. In fact, the elongating enzyme appeared to transcribe faster under the extreme conditions of high KCl concentrations and elevated temperature.

Block to Elongation Occurring at High Temperatures Is Irreversible. The following experiment was performed in order to characterize the nature of the elongation block at the SV40 attenuator 1 which occurs at high temperature. The transcription elongation reaction was carried out in 0.2 M KCl at 65 °C for 15 min. At this time point, about 50% of the
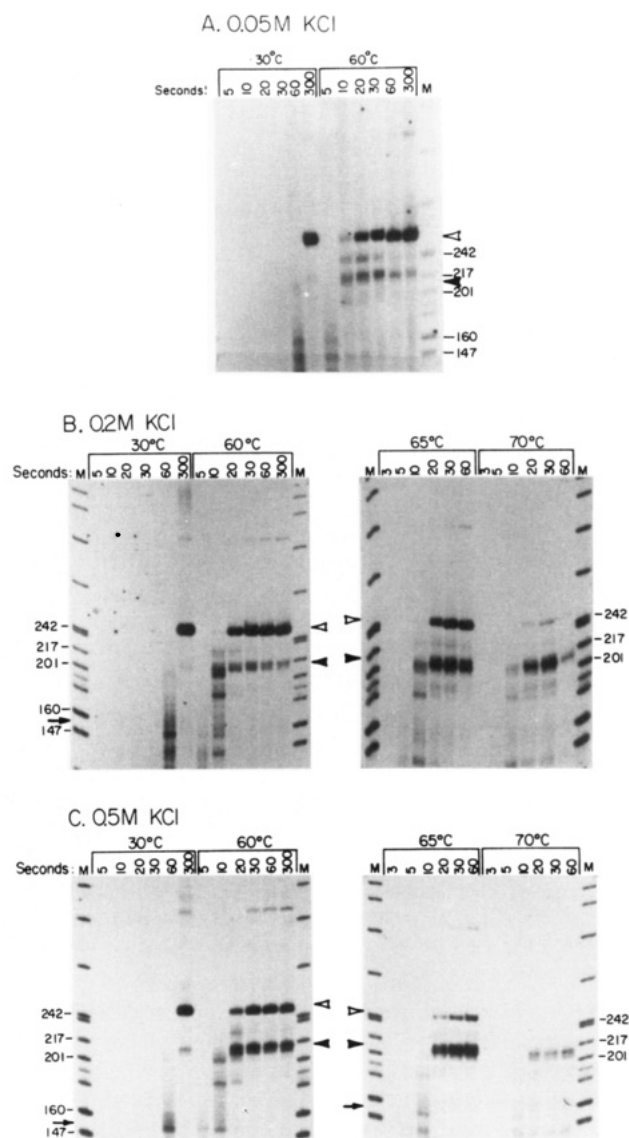
FIGURE 7: Comparison of the elongation kinetics in various KCl concentrations and at different temperatures using the WT construct. Preincubation and transcription initiation were performed as described in Figure 3. Elongation was performed in 0.05 M KCl (panel A), 0.2 M KCl (panel B), and 0.5 M KCl (panel C) at the temperatures and times indicated. The runoff (open arrowhead) and the attenuator RNAs (solid arrowhead) are indicated.
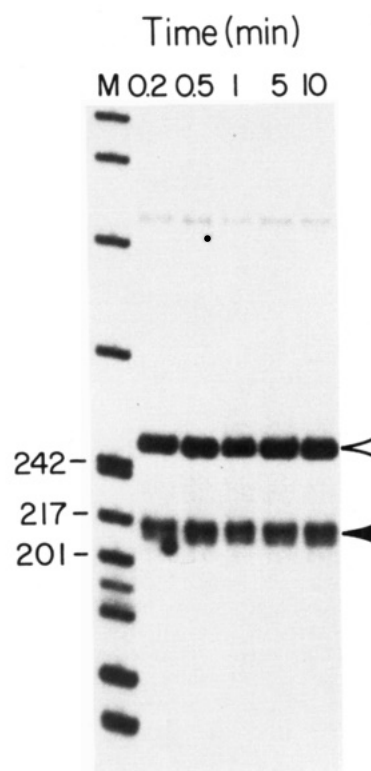

FIGURE 8: Transcription elongation on the WT construct at high temperature followed by a shift to low temperature. Preincubation and transcription initiation were performed as described in Figure 3. Elongation in 0.2 M KCl at 65 °C for 15 min was followed by continued incubation at 30 °C for times as indicated. Size analysis of the RNA produced was done as described under Materials and Methods. The runoff (open arrowhead) and the attenuator RNAs (solid arrowhead) are indicated.

polymerase molecules are blocked at the attenuation site, giving rise to the synthesis of the attenuator RNA. The reaction was then shifted to 30 °C, a temperature where the only RNA synthesized is the runoff RNA (see Figure 7, panel B), and samples were analyzed several times thereafter. Figure 8 shows that the relative amount of the attenuator RNA as compared to the runoff RNA was unchanged, indicating that the polymerase is either irreversibly blocked or released at the SV40 attenuator 1. It is worth noting, however, that although the transcription complexes elongate efficiently at 65 °C (see Figure 7), the possibility that the attenuated transcription complexes which are arrested at a specific site have lost their viability has not been excluded.

General Elongation Factors IIS, IIF, and IIX Are Not Involved in Modulating the Extent of the Elongation Block at the SV40 Attenuator 1. The accumulation of attenuated transcripts at high concentration of KCl could be due to the dissociation of elongation factors from the transcription complexes, inactivation of the elongation factors, or changes in the structure but not content of the complex. These possibilities can be distinguished from each other by initiating transcription in the absence of GTP, to generate template containing short nascent RNA transcripts, partially purifying the template by Sephacryl S-1000 gel filtration in 0.8 M KCl, and then elongating the nascent transcripts at different concentrations of KCl. If the chromatography at high KCl concentration removes the elongation factors which prevent transcription attenuation, the purified complexes will synthesize the attenuator RNA even when the KCl during elongation is diluted to 0.05 M (Bengal et al., 1989, 1991). If, on the other hand, the high KCl concentration directly affects the structure but not the content of the elongating complex, then passage of the briefly initiated complexes through the column at 0.8 KCl should have no effect, and the attenuator RNA should be synthesized to the same extent as with nonpurified complexes (i.e., only at high KCl concentration). We have recently used this approach to show that the synthesis of the Ad2 attenuator RNA is modulated by the cellular transcription factors IIS, IIF, and IIX (Bengal et al., 1989, 1991). As can be seen in Figure 9, the synthesis of the attenuator RNA on the column-purified complexes occurred as in the latter prediction, i.e., when the KCl concentration during elongation was above 0.5 M. These results indicate that, in contrast to the mechanism of attenuation at the Ad2 attenuator (Bengal et al., 1991), SV40 attenuator 2 (Kessler et al., 1991), and MVM attenuator (Krauskopf et al., 1989), using the Ad2/SV40 chimera the general elongation factors IIS, IIF, and IIX are not directly involved in modulating the elongation block at the SV40 attenuator 1. It is worth noting that the present experiments do not provide direct evidence that no cellular factors function in this process. It is possible, for example, that an elongation factor could remain associated
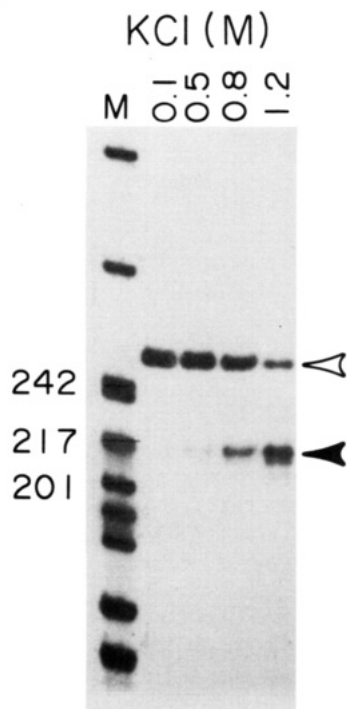
KCl (M)

FIGURE 9: Size analysis of the RNA produced from transcription complexes chromatographed on a Sephacryl S-1000 column at 0.8 M KCl. Preincubation and transcription initiation of the equivalent of eight reactions were performed as described in Figure 3. Briefly, initiated transcription complexes were passed through a Sephacryl-1000 column equilibrated with 0.8 M KCl. The first four fractions containing radioactivity from the void volume were pooled (Bengal et al., 1991) and allowed to further elongate the nascent RNA under the standard chase conditions (as described in Figure 3) containing different concentrations of KCl as indicated. The runoff (open arrowhead) and the attenuator RNAs (solid arrowhead) are indicated.

with the elongation complex at high salt even though its proper function is inhibited under these conditions.

DISCUSSION

We have previously shown that RNA polymerase II initiating transcription in vivo or in vitro at the SV40 major late initiation site can terminate in vitro 95 nt downstream and that the attenuator RNA can fold into a hairpin structure followed by U residues, resembling a typical prokaryotic ρ-independent terminator. The attenuator RNA can also fold into an alternative hairpin which is not followed by U residues [for a review, see Aloni and Hay (1985)]. We have suggested that the decision whether the RNA polymerase will prematurely terminate and procuce the 95-nt attenuator RNA or will continue and produce the full-length primary transcript depends on which of the alternative conformations at the 5′-end of the RNA transcript prevails (see Figure 1A,B for the two alternative conformations) (Hay et al., 1982; Aloni & Hay, 1985). The results of the present study fully support the involvement of the predicted secondary structures in transcription attenuation and indicate striking similarity to the mechanism of transcription attenuation by *E. coli* RNA polymerase. We have also shown that RNA polymerase II (in cell-free extract) recognizes the SV40 sequences that lead to the elongation block even when they are placed following the Ad2 MLP. The extent of the elongation block is dependent on the stability of the attenuator RNA secondary structure, as assessed by transcription of three mutated templates in HeLa WCE in the presence of high KCl concentrations (0.5–1.2 M). Similar results were previously obtained when transcription elongation in HeLa WCE was performed in the

presence of 0.3% Sarkosyl at 45 °C (Resnekov et al., 1989). It is worth noting that the present results do not provide evidence to show that the (2+3) readthrough conformation (see Figure 1B) is involved in antitermination.

We can envisage three ways by which high KCl concentrations during elongation enhance attenuation: (1) through inactivation of elongation factors or dissociation of elongation factors from the elongating complex; (2) through stabilization of RNA secondary structures; (3) through protein–nucleic acid and protein–protein interactions.

It is unlikely that the inactivation or the removal of the elongation factors IIS, IIF, and IIX (Bengal et al., 1991) from the transcription complex by high KCl concentrations leads to elongation arrest at the attenuation site, since inactivation or removal of loosely bound elongation factors from the elongation complex by treatment with 0.8 KCl followed by gel filtration did not result in the accumulation of attenuated transcripts with continued elongation at low KCl concentration. Transcription attenuation with the column-purified complexes, as with nonpurified complexes, was dependent on the presence of high concentrations of KCl during elongation (see Figure 9). Thus, in contrast to the Ad2 attenuator (Bengal et al., 1989, 1991), SV40 attenuator 2 (Kessler et al., 1991), or MVM attenuator (Krauskopf et al., 1991), the general elongation factors IIS, IIF, and IIX are not directly involved in modulating the elongation block at the SV40 attenuator 1. Instead, it is possible, as we have previously suggested (Hay et al., 1982; Aloni & Hay, 1985), that the viral proteins T-Ag and agnoprotein are involved in modulating the elongation block at the SV40 attenuator 1. The second possibility—that the high KCl concentrations simply stabilize the RNA secondary structure—is feasible because the level of the elongation block was in direct correlation with the stabilities of the RNA secondary structures. However, our previous observation of a similar effect on the efficiency of transcription attenuation when elongation was carried out in the presence of 0.3% Sarkosyl at 45 °C (Resnekov et al., 1989), conditions which do not affect the stabilization of RNA secondary struture, may indicate that stabilization of RNA secondary structure is only part of the effect. Alternatively, it is possible that there are two different mechanisms which could lead to the same effect on transcription attenuation. The third possibility—that different KCl concentrations alter the protein–nucleic acid and/or protein–protein interaction—seems most likely and can be explained by the effect of Sarkosyl at 45 °C and that of high KCl concentrations. The second and third possibilities can also be reviewed in the framework of the models which are now being debated concerning termination at a ρ-independent terminator ("intrinsic terminator") in prokaryotes (Reynolds et al., 1992; von Hippel & Yager, 1991; Yager & von Hippel, 1991).

In a recent study, utilizing synthetic attenuators, transcribed by the eukaryotic RNA polymerase II under the control of the Ad2 MLP, we have shown that, as in the prokaryotic system, the extent of the elongation block is directly correlated to the stability of the stem- and loop structure and the number of U residues following them (Bengal & Aloni, 1989). Furthermore, transcription of the HIV attenuator in a dC-tailed template has indicated that the recognition of a stem-loop structure as a termination signal is an intrinsic property of RNA polymerase II (Bengal & Aloni, 1991). We therefore conclude that RNA polymerase II can recognize RNA secondary structure as a signal for an elongation block. It should, however, be noted that in other studies (Kessler et al.,

1989; Resnekov et al., 1991), although the attenuator RNA can potentially fold into secondary structure, experiments have indicated that in these cases RNA secondary structure is not the dominant element. Thus, not in each system where RNA secondary structure can potentially be formed does it function as a dominant element in transcription attenuation. In support of the notion that the U stretch has an important function in attenuation by the eukaryotic polymerase II, we have shown in the present study that increasing the temperature during elongation enhanced the percent attenuation, presumably by destabilizing the rU–dA interactions (alternatively, the increased temperature can affect protein–nucleic acid or protein–protein interactions), and in our recent studies with Ad2 attenuator and synthetic attenuators, we have observed a direct relationship between the length of the U stretch and the percent termination (Kessler et al., 1989; Bengal & Aloni, 1989).

Chamberlin and co-workers, in analyzing transcription of ρ-independent termination by *E. coli* polymerase, have proposed that although formation of a stable RNA hairpin is clearly a sine qua non of terminator function, the notion of a simple thermodynamic relationship between percent termination and RNA hairpin stability cannot be supported (Reynolds et al., 1992). In support of their argument, they refer to the recent finding that the actual sequence in the stem which is mutated to destabilize the RNA terminator hairpin can have a great effect on the percent termination in vivo (Reynolds et al., 1992). They suggest that the enzyme must play a major catalytic role in determining the efficiency of termination and the termination process must consist of several distinct steps (Reynolds et al., 1992). In support of this model, it has been observed that mutations in the RNA polymerase β subunit can dramatically enhance or reduce termination at ρ-independent terminators in vitro (Neff & Chamberlin, 1980; Yanofsky & Horn, 1981). A similar type of model could be employed to explain our studies concerning transcription through potential attenuation sites in the presence of 0.3% Sarkosyl at 45 °C and at high KCl concentrations.

We have previously reported that RNA polymerase II has both unwindase and rewindase activities which define the limits of the DNA unwound region and catalyze its propagation through the double helix (Choder & Aloni, 1988). Similar activities were assigned to the *E. coli* polymerase (von Hippel et al., 1984). The common activities of *E. coli* and RNA polymerase II enzymes, the similar features of the transcription bubble (Choder & Aloni, 1988), and the extensive amino acid homology shared by the β-elongating subunit of *E. coli* RNA polymerase and several eukaryotic elongating subunits of RNA polymerases II (Allison et al., 1985, 1988; Biggs et al., 1985; Broyles et al., 1986; Sweetser et al., 1987) support the notion that the eukaryotic and prokaryotic polymerases can respond to similar pausing and termination signals (Resnekov et al., 1988). We therefore suggest that the mechanism by which RNA polymerase II terminates transcription at the SV40 attenuator 1 is similar to that of ρ-independent termination by *E. coli* RNA polymerase.

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REFERENCES

Allison, L. A., Moyle, M., Shales, M., & Ingles, C. J. (1985) *Cell* 42, 599–610.

Allison, L. A., Jerry, K.-C., Wong, V., Fitzpatrick, D., Moyle, M., & Ingles, J. (1988) *Mol. Cell. Biol.* 8, 321–329.

Aloni, Y., & Hay, N. (1985) *CRC Crit. Rev. Biochem.* 18, 327–383.

Bengal, E., & Aloni, Y. (1989) *J. Biol. Chem.* 264, 9791–9798.

Bengal, E., & Aloni, Y. (1991) *J. Virol.* 65, 4910–4918.

Bengal, E., Goldring, A., & Aloni, Y. (1989) *J. Biol. Chem.* 264, 18926–18932.

Bengal, E., Flores, O., Krauskopf, A, Reinberg, D., & Aloni, Y. (1991) *Mol. Cell. Biol.* 11, 1195–1206.

Biggs, J., Searles, J., & Greenleaf, A. L. (1985) *Cell* 42, 611–621.

Broyles, S. S., & Moss, B. (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83, 3141–3145.

Choder, M., & Aloni, Y. (1988) *J. Biol. Chem.* 263, 12994–13002.

Dedrick, R. L., Kane, C. M., & Chamberlin, M. J. (1987) *J. Biol. Chem.* 262, 9098–9108.

Farnham, P. J., & Platt, T. (1980) *Cell 20*, 739–748.

Hay, N., & Aloni, Y. (1984) *Nucleic Acids Res.* 12, 1401–1414.

Hay, N., Skolnik-David, H., & Aloni, Y. (1982) *Cell 29*, 183–193.

Kessler, M., & Aloni, Y. (1989) *Gene 84*, 65–72.

Kessler, M., Ben-Asher, E., & Aloni, Y. (1989) *J. Biol. Chem.* 264, 9785–9790.

Kessler, M., Ben-Asher, E., Resnekov, O., Hatini, V., Bengal, E., & Aloni, Y. (1991) *J. Biol. Chem.* 266, 13019–13027.

Krauskopf, A., Bengal, E., & Aloni, Y. (1991) *Mol. Cell. Biol.* 11, 3515–3521.

Manley, J. L., Fire, A., Cano, A., Sharp, P. A., & Gefter, M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 3855–3859.

Martin, F. H., & Tinoco, I., Jr. (1980) *Nucleic Acids Res. 8*, 2295–2299.

Neff, N. F., & Chamberlin, M. J. (1980) *Biochemistry 19*, 3005–3015.

Pfeiffer, P., Hay, N., Pruzan, R., Jakobovits, E. B., & Aloni, Y. (1983) *EMBO J. 2*, 185–191.

Platt, T. (1981) *Cell 24*, 10–23.

Platt, T. (1986) *Annu. Rev. Biochem.* 55, 339–372.

Resnekov, O., & Aloni, Y. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86, 12–16.

Resnekov, O., Ben-Asher, E., Bengal, E., Choder, M., Hay, N., Kessler, M., Ragimov, N., Seiberg, M., Skolnik-David, H., & Aloni, Y. (1988) *Gene 72*, 91–104.

Resnekov, O., Kessler, M., & Aloni, Y. (1989) *J. Biol. Chem.* 264, 9953–9959.

Resnekov, O., Pruzan, R., & Aloni, Y. (1991) *Nucleic Acids Res. 19*, 1783–1790.

Reynolds, R., Bermudez-Cruz, R. M., & Chamberlin, M. J. (1992) *J. Mol. Biol.* 224, 31–51.

Rosenberg, M., & Court, D. (1979) *Annu. Rev. Genet. 13*, 319–353.

Skolnik-David, H., & Aloni, Y. (1983) *EMBO J. 2*, 179–184.

Skolnik-David, H., Hay, N., & Aloni, Y. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2743–2747.

Sweetser, D., Nonet, M., & Young, R. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1192–1196.

von Hippel, P. H., & Yager, T. D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2307–2311.

von Hippel, P. H., Bear, D. G., Morgan, W. D., & McSwigger, J. A. (1984) *Annu. Rev. Biochem. 53*, 389–446.

Yager, T. D., & von Hippel, P. H. (1987) in *Transcript Elongation and Termination in Escherichia coli and Salmonella typhimurium* (Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., & Umbarger, H. E., Eds.) p 1241, American Society for Microbiology, Washington, D.C.

Yager, T. D., & von Hippel, P. H. (1991) *Biochemistry 30*, 1097–1118.

Yanofsky, C., & Horn, V. (1981) *J. Bacteriol. 145*, 1334–1341.