Reiterative copying by *E.coli* RNA polymerase during transcription initiation of mutant pBR322 *tet* promoters

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ABSTRACT

The major *in vitro* transcripts from the *tet* promoter of pBR322 derivatives pTA22 and pTA33 have heterogeneous 5′ ends consisting of variable lengths of oligo(A). Their structure is 5′pppA$_n$U···, where n ranges from 1 to >12, but the template strand can encode at most four A residues at the site of transcription initiation. The abundance of additional A residues at the 5′ end of the pTA22 and pTA33 *tet* transcripts could be reduced by elevating the concentration of UTP, but even at high concentrations (>1 mM) non-cognate A residues were still observed. Aberrant initiation was not artifactual since the major and minor transcripts of the pBR322 *tet* promoter region, and other transcripts arising from minor promoters on pTA22 or pTA33 DNA all had unique 5′ termini. Mixing experiments showed that RNA polymerase did not utilize pppA$_{2-4}$-OH produced by abortive initiation as primers. The data suggest that the initial nascent RNA chain 'slips' in the 5′ direction during elongation opposite T$_4$ on the template strand causing RNA polymerase to reiteratively add A residues to the 5′ end of the transcript. The generality and possible significance of this mechanism is discussed.

INTRODUCTION

RNA polymerase initiates transcription at sites in DNA specified by promoter sequences. In *E. coli*, promoters are well characterized by two conserved sequences, the −35 region and the Pribnow Box, or −10 region, centered approximately 35 and 10 basepairs (bp), respectively, upstream of the start point of transcription initiation (see 1,2). The exact base(s) at which transcription initiates, however, is difficult to predict. In part, this arises because the 5′ end of the primary transcript of genes is often determined by S1 mapping or primer extension studies which do not identify the *exact* 5′ nucleotide and thus limit theoretical studies attempting to identify signals for transcription initiation (2). In addition, the 5′ end of the primary transcript often appears heterogeneous, which has usually been interpreted to indicate multiple start points or limitations of the experimental method.

In a previous report we have shown that *tet* transcripts from two pBR322 derived plasmids containing AT-rich inserts within the promoter region are heterogeneous at their 5′ end (3). Heterogeneity results from variable lengths of 5′ oligo(A) sequences extending beyond the possible range encoded by the DNA template. Such anomalous behaviour of RNA polymerase was first reported for artificial single-stranded templates (4−6), but our data, and those of others (7−10), suggest that it may also occur during initiation from normal promoters opposite homonucleotide stretches on the template strand. We report studies on the characteristics of reiterative copying by RNA polymerase from these *tet* gene promoters and discuss its possible significance.

MATERIALS AND METHODS

In vitro transcription

HaeIII restriction fragments of size 192 bp (pBR322), 214 bp (pTA22) or 225 bp (pTA33) were isolated from plasmids pBR322, pTA22 and pTA33 for transcription *in vitro* as described previously (3), except that α-[^{32}P]UTP, γ-[^{32}P]ATP, or γ-[^{32}P]GTP were used as radiolabeled nucleotide precursors. These fragments extended from 4345 to 174 on the pBR322 map (12), encompassing the *tet* promoter region from 63 bp upstream of the major transcription start of each plasmid to a point 130−160 bp into the transcribed region (3). *E. coli* RNA polymerase was prepared as described (11). In some experiments, commercial RNA polymerase (New England Biolabs, MA) was used with essentially identical results. Except where indicated, standard transcription reactions involved incubation of 0.3−0.5 pmol DNA with 2 pmol RNA polymerase at 37° C for 30 min with 150 μM of each ribonucleoside triphosphate.

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RNA characterization

Transcripts uniquely labeled at their 5′ end were resolved by acrylamide gel electrophoresis, eluted, and purified as described previously (3). Labeled RNA (200–5000 cpm) was mixed with 2 μg wheat germ tRNA and partially or completely digested with pancreatic RNase A or RNase T1 (13,14). Products were resolved on 20% acrylamide, 7 M urea gels and visualized by autoradiography. The known sequence of the template DNA allowed unambiguous identification of transcription start points from the pattern of partial cleavage products. In some experiments, start points were identified by RNA fingerprinting (3). Figures shown represent typical autoradiograms from replicate or triplicate experiments.

Quantitation.

Autoradiograms exposed within the linear range of the film without intensifying screens were scanned with a densitometer and the area corresponding to each radioactive band was integrated. Relative abundance of RNA was assumed to be proportional to the area of corresponding peaks from the densitometer scan.

RESULTS AND DISCUSSION

Heterogeneous Start of *tet* Transcripts from pTA22 and pTA33

Plasmids pTA22 and pTA33 were derived from pBR322 by insertion of AT-rich oligonucleotides (22 and 33 bp in length, respectively) at the HindIII site within the *tet* promoter of pBR322. All three plasmids confer significant tetracycline resistance to transformed cells, and the major *in vitro* transcripts from HaeIII fragments encompassing the *tet* promoter have been identified (Fig. 1, see also ref. 3). The level of transcription from pTA22 and pTA33 plasmids was greater than that from pBR322, as expected from previous reported experiments (3). However, upon sequencing these transcripts, the 5′ ends were found to consist of a variable number of A residues (up to 12 or more, with a mode of 5 or 6) (Fig. 1B, also ref. 3). This tract of 5′ oligo(A) cannot be encoded by the template strand by the usual mechanism since at most A_4 would be specified at the point of initiation. It is unlikely that these extraneous residues arise as an artifact of the *in vitro* transcription system, since the only major *tet* transcripts from pTA22 and pTA33 consistently had 5′-oligo(A) tails, and no other transcript which we studied, including minor transcripts from these DNA fragments or from other plasmids, had anomalous 5′ ends (not shown). Due to the relatively low level of *tet* transcripts *in vivo*, we were unable to test for heterogeneity at the 5′ end of *tet* mRNA in transformed *E. coli*.

Similar results have been reported by others for *E. coli* RNA polymerase transcripts arising from specific promoters. For example, bacteriophage fd promoter I initiates transcription with 5′ oligo(G) opposite C_3 on the template strand (7,8), the leftward promoter of IS1 initiates with 5′ oligo(U) opposite A_5 on the template (9), P_{82} lambda promoter generates 5′ oligo(A) opposite A_3 on the template (Jeff Roberts, personal communication), and, in the most extreme case, a mutated *sar* promoter (in which the +1 site was changed from TGTT to TTTT) caused synthesis of poly(U) only (J.-P. Jacques and M. Susskind, personal communication). Synthetic T7 promoters also permit poly(G) synthesis opposite G_3 by T7 polymerase (10). In some cases reiterative copying was only observed when a single ribonucleoside triphosphate (the initiating ribonucleotide) was present. However, the common feature of these promoters is initiation of transcription within a homonucleotide stretch on the template strand.

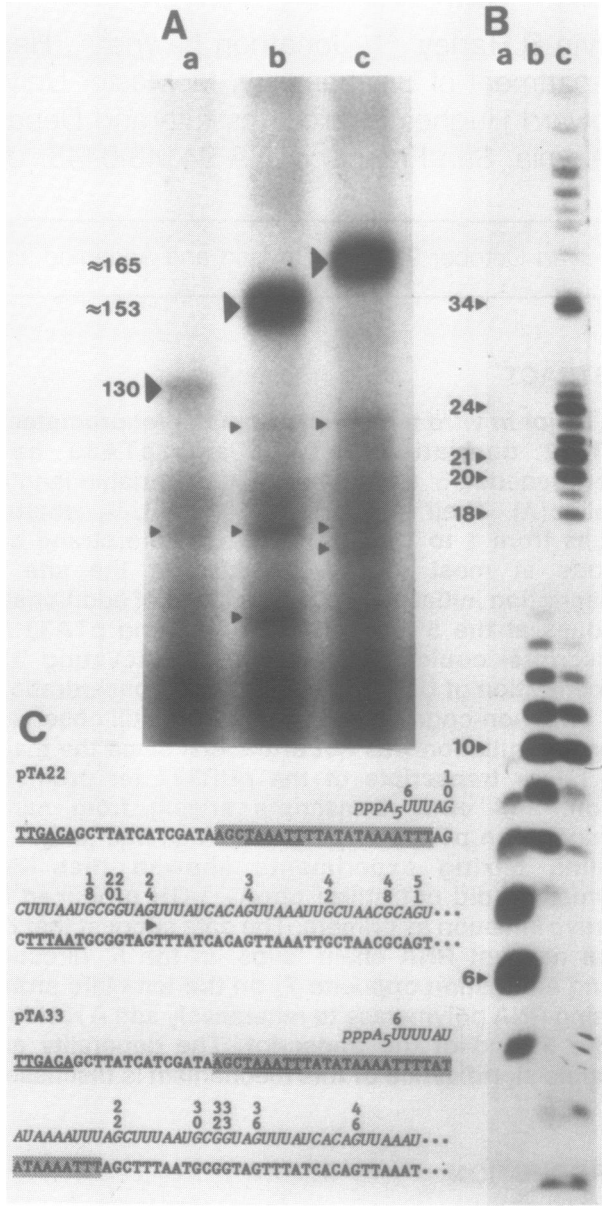

Figure 1. **(A)** *In vitro* transcripts from HaeIII fragments containing the *tet* gene promoter of pBR322 **(a)**, pTA22 **(b)** and pTA33 **(c)**. Transcripts were labeled with α-[^{32}P]UTP **(A)** as described in Materials and Methods and resolved by electrophoresis through a 5% denaturing polyacrylamide gel. A typical gel is shown with major and minor transcripts indicated with large and small arrowheads, respectively. Numbers indicate approximate run-off transcript size. **(B)** RNase digestion of the major γ-[^{32}P]ATP-labeled transcript from the pTA22 *tet* gene fragment. RNA was isolated from gels and digested to completion with pancreatic RNase A **(a)** or T1 **(b)**. Lane **c** shows a partial digestion pattern obtained with RNase T1. Fragments were resolved on a 20% denaturing polyacrylamide gel. Numbers correspond to nucleotides from the 5′-end of the pTA22 transcript as indicated in **(C)**. **(C)** Sequence of pTA22 and pTA33 *tet* promoter region and 5′-end of the major transcripts. The sequence inserted at the HindIII site of pBR322 is highlighted. Minus 35 and −10 regions are doubly underlined. Numbers above the RNA sequence assume a $pppA_5U\cdots$ start and indicate positions of the first pancreatic RNase A site and all RNase T1 sites in the first 50 bases. The pBR322 *tet* −10 region and transcription start are indicated by a single underline and arrowhead, respectively, on the pTA22 sequence.

Models

The mechanism of reiterative copying by RNA polymerase has not been extensively investigated. Heyden et al. (7) speculated that RNA polymerase might use preformed oligonucleotides as primers (Fig. 2A), while others (eg. 9,10) assumed a slippage mechanism similar to the model proposed by Chamberlain and Berg (5) (Fig. 2B). In the latter model, reiterative copying presents an alternative to abortive initiation in which the release of short nascent transcripts, presumably before sigma factor dissociates, allows the polymerase to maintain contact with the promoter and reinitiate RNA synthesis (15, 16). The DNA footprint of the initial transcribing complex suggests that RNA polymerase does not, in fact, change conformation until it has moved past the +10 position, approximately, of the template strand (16). If sequence redundancy allowed the RNA/DNA heteroduplex to slip without complete dissociation, then the transcript could be extended rather than prematurely terminated. To further investigate this phenomenon in transcription from pTA22 and pTA33 *tet* promoters and to discriminate between these models, we conducted the following experiments.

Effect of Precursor Concentrations and Temperature

The effect of UTP, ATP, and temperature on generation of 5′-oligo(A) from the pTA22 or pTA33 *tet* promoter is shown in Fig. 3. Increased UTP concentration decreases the abundance of longer oligo(A) stretches in the transcript (Fig. 3A), which might be expected if slippage is blocked by incorporation of U followed by normal chain extension after the first few A residues were added to the nascent RNA. In contrast to the efficient inhibition of reiterative cycling by sub-micromolar concentrations of UTP reported by Chamberlain and Berg (5), 5′-oligo(A) was observed with pTA22 and pTA33 promoters in the presence of standard UTP concentrations (100−150 μM) and was only partially inhibited by concentrations up to 1 mM (Fig. 3A). Additional 5′ G residues caused by apparent reiterative copying from a phage fd promoter were also only partially suppressed by physiological concentrations of the other ribonucleoside triphosphates (8).

It is also possible that increased UTP reduces the frequency of abortive initiation from these *tet* promoters and hence reduces the concentration of $pppA_n$ primers which could be generated by cycling of the polymerase. We in fact observed a shift in the distribution of 5′$pppA_n$ towards shorter lengths when UTP was added to reactions which otherwise contained only ATP (Fig. 3B). Therefore, these data do not exclude use of preformed primers by RNA polymerase in generating 5′-oligo(A).

With increasing ATP concentrations, the relative frequency of full-length transcripts which contained 5′-$pppA_n$, $n>4$, increased at the expense of cognate transcripts (Fig. 3C, Table 1).

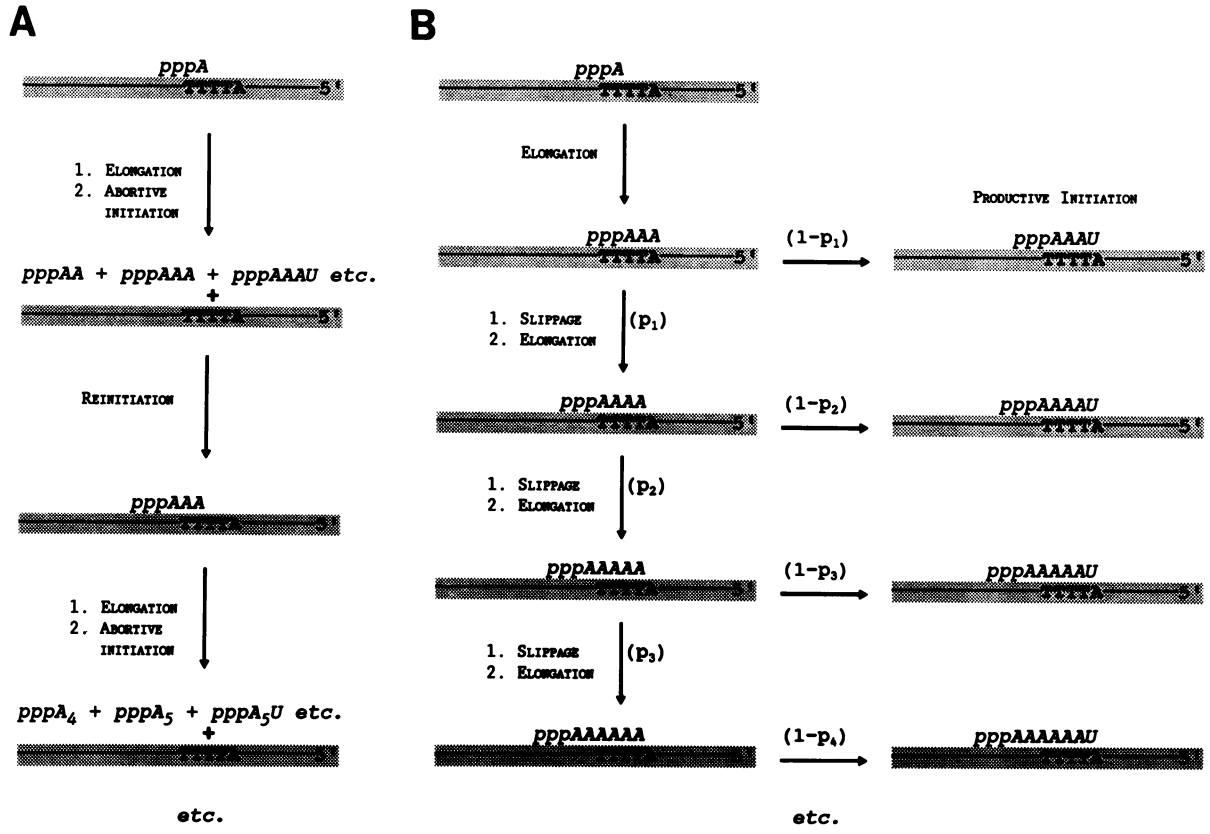

Figure 2. Alternative models explaining reiterative copying by RNA polymerase. In the cases illustrated transcription initiates at the second position in the string of T residues on the highlighted template strand. Similar models could be drawn for initiation at any of the first three T residues. **(A)** Rounds of partial elongation and abortive initiation release $pppA_n$ which are used to prime initiation of subsequent cycles. At any step, productive initiation by addition of a U residue and normal elongation competes with abortive initiation. Thus full-length transcripts contain $pppA_nU\cdots$ from abortive initiation products at their 5′ ends. **(B)** Slippage of $pppA_3$, $pppA_4$, *etc.* on the template strand, presumably driven by more favourable interactions between the polymerase and the promoter, generate 5′ oligo(A). Productive initiation competes with reiterative copying as in **(A)**. Abortive initiation is not excluded in model **(B)**, but aborted transcripts are not incorporated into full-length transcripts. Probabilities of slippage (p_1, p_2, p_3, *etc.*) are discussed in reference to Table 1.

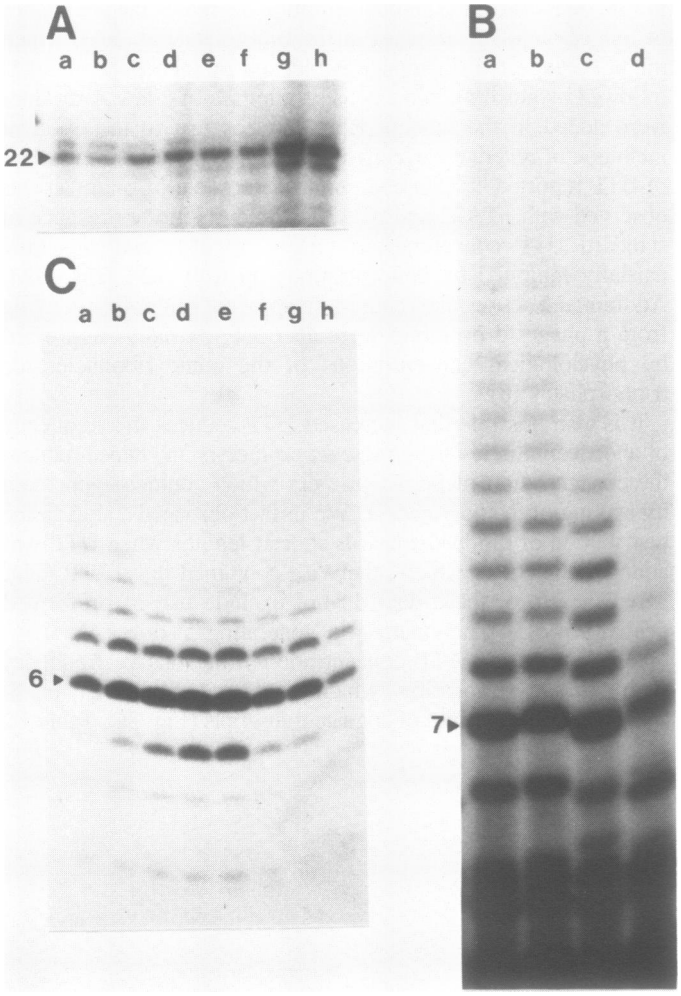

Figure 3. Effect of precursor concentrations and temperature on reiterative cycling. **(A)** The *tet* gene fragment from pTA33 was transcribed as described in Fig. 1 except the UTP concentration was 40 μM **(a,b)**, 130 μM **(c,d)**, 300 μM **(e,f)** or 1.2 mM **(g,h)**. Other ribonucleoside triphosphates were 150 μM. Transcripts were digested to completion with RNase T1. The major band (arrowhead) corresponds to $pppA_5U_4AUAUA_4U_3AGp$. **(B)** Abortive initation products from pTA33 *tet* fragment transcription with 150 μM ATP alone **(a,b)** or with 150 μM ATP and UTP **(c,d)**. Products from the transcription reaction were undigested **(a,c)** or digested to completion with pancreatic RNase A **(b,d)**. Bands at position '7' likely correspond to $pppA_6$-*OH* **(a,b)**, $pppA_5U$-*OH* **(c)**, and $pppA_6Up$ **(d)**. **(C)** The *tet* gene fragment from pTA22 was transcribed as described in Fig. 1 with the following modifications: the temperature of the reaction was 18 °C **(a)**, 23 °C **(b)**, 30 °C **(c)** or 37 °C **(d)**; the ATP concentration was 100 μM **(e)**, 200 μM **(f)**, 400 μM **(g)** or 800 μM **(h)**. Transcripts were isolated and digested to completion with pancreatic RNase A. The major band (arrowhead) corresponds to $pppA_5Up$. All transcripts in **A,B,C** were labeled with γ-[^{32}P]ATP during transcription and resolved on a 20% denaturing polyacrylamide gel.

The decrease in total transcript signal with increasing ATP concentration (Fig. 3C) reflects the decreased specific activity of γ-[^{32}P]ATP and not inhibition of transcription. These results could be consistent with either model for generation of 5′-oligo(A). However, the data are more easily reconciled with the slippage model since it is expected that ATP would compete with abortive initiation products for priming *tet* gene transcription. If the primer model were correct, increased levels of ATP added to the reaction would decrease, rather than increase, the frequency of 5′-oligo(A).

If RNA polymerase slips during initial elongation of the nascent transcript, hydrogen bonds must be broken as the RNA:DNA heteroduplex shifts. This component of the process would be inhibited at lower temperatures since the heteroduplex would be more stable. As shown in Fig. 3C, anomalously extended transcripts actually occur with higher frequency at lower temperatures. However, this does not provide strong evidence against the slippage model since interactions between RNA polymerase and the promoter which presumably drive slippage might also be stronger at lower temperatures and more than compensate for the increased stability of the short RNA:DNA heteroduplex.

In other experiments, concentrations of potassium chloride and magnesium acetate in the reactions were varied within normal ranges for transcription (100−160 mM and 2−5 mM, respectively) but no effect on 5′-heterogeneity was observed (not shown). Similarly, different sources of RNA polymerase produced essentially identical results.

RNA polymerase does not use preformed primers

We wished to test directly whether RNA polymerase uses preformed abortive oligo(A) initiation products to generate the observed 5′-heterogeneity of pTA22 and pTA33. Transcription was initiated on the pTA22 promoter fragment and after several minutes to allow labeled abortive initiation products to accumulate, the promoter fragment from pTA33 was added with excess cold UTP (as a control) or cold ATP (to dilute the radiolabeled ATP) and the reaction continued for 25 min. Run-off transcripts (Fig. 4A) as well as T1 digestion products (Fig. 4B) from pTA22 and pTA33 were resolved by gel electrophoresis. The 5 min preincubation with pTA22 fragment is sufficient to generate full-length pTA22 transcript (lane a, Fig. 4A). If preformed primers contributed significantly to transcription, then radioactive transcripts of pTA33 would be formed equally in the presence of cold UTP or cold ATP. The data shown in Fig. 4 show that RNA polymerase did not utilize labeled $pppA_n$ fragments generated during abortive initiation as primers. Instead, radioactive pTA33 transcripts with heterogeneous 5′-termini were made only in the absence of excess cold ATP (Fig. 4B, lane b and 4C, lane b), indicating that the pTA33 product arises predominantly from *de novo* extension of ATP used to initiate transcription. It is possible that the absence of radioactive pTA33 product reflects in part competition of cold ATP with preformed primers. However, this seems unlikely since in the experiment described in Fig. 3C, increasing the cold ATP concentration increased, rather than decreased the frequency and extent of 5′-oligo(A) sequences. Finally, when heparin was added 10 minutes after initiation of transcription to prevent reinitiation of RNA polymerase, no effect on 5′-oligo(A) was observed (not shown). Thus, these data show that the 5′-heterogeneity of transcripts from the pTA22 and pTA33 *tet* promoters do not arise from reinitiation and extension of preformed primers. The reduced heterogeneity of the product pTA33 product seen in Figure 4B, lane b, reflects the effect of excess UTP in the reaction as discussed above (see Fig. 3A).

Quantitation

To allow quantitative comparison of the degree of reiterative copying by RNA polymerase under different conditions, a simple calculation was done from scans of autoradiograms to estimate the probability of slippage versus normal elongation (Fig. 2 and Table 1). Our approach ignores kinetic effects and differential stability of transcripts. A more complex model did not seem warranted by the data. A 1-parameter Poisson distribution with

TABLE 1

Frequency of Transcripts (%)

	TEMPERATURE (°C)								[ATP] (μM)							
	18		23		30		37		100		200		400		800	
	O	E	O	E	O	E	O	E	O	E	O	E	O	E	O	E
5'-END																
A_3U	3	3	2	2	3	3	4	4	2	2	3	3	3	3	1	1
A_4U	5	5	6	6	12	12	17	17	18	18	9	9	6	6	4	4
A_5U	54	55	63	64	65	64	59	59	62	62	66	66	62	63	48	49
A_6U	18	22	19	20	16	16	14	15	13	14	16	17	19	20	23	24
A_7U	6	9	5	6	3	4	3	4	2	3	4	4	4	6	8	12
A_8U	5	4	3	2	1	1	1	1	1	1	2	1	2	2	6	6
A_9U	5	2	1	1			1	0	1	0	1	0	1	0	4	3
$A_{10}U$	2	1													4	1
$A_{11}U$															1	0
p_1	0.97		0.98		0.97		0.96		0.98		0.97		0.97		0.99	
p_2	0.95		0.94		0.88		0.82		0.82		0.91		0.94		0.96	
p_3	0.40		0.31		0.25		0.25		0.22		0.25		0.31		0.49	

Shown are observed (O) and expected (E) frequencies of run-off transcripts having indicated 5'-ends for the data described in Fig. 3C. Probabilities p_1, p_2, p_3 of slippage used to calculate expected frequencies are also shown. Observed frequencies were determined by quantitative scans of autoradiograms.

a fixed probability of reiterative copying was not consistent with observations since the frequency of $pppA_3U\cdots$ and $pppA_4U\cdots$ transcripts were both less than that of $pppA_5U\cdots$ transcripts. It was clear that the probability of slippage at $pppA_3$ and $pppA_4$ (p_1 and p_2, respectively, Fig. 2) were quite high compared to subsequent slippage events. Therefore, we allowed probabilities p_1 and p_2 to be determined from measured frequencies of $pppA_3U\cdots$ and $pppA_4U\cdots$ transcripts and then tested whether a third probability p_3 was sufficient to predict the frequencies of all other transcripts ($pppA_nU\cdots$, $n>4$). This probability was determined by minimizing the variance between expected and observed frequencies for $pppA_nU\cdots$ ($n>4$). In most cases densitometry permitted quantitation of transcripts only up to $pppA_9U\cdots$ or $pppA_{10}U\cdots$ and over this range theory and data were in good agreement (Table 1): none of the frequency distributions was statistically inconsistent with the model ($p >> 0.1$; chi-squared test). Whether the model is applicable at higher levels of reiterative copying, or with other promoters is not known. For example, other factors are clearly important in reiterative copying by T7 polymerase from synthetic promoters since 5' homonucleotide extension appears to abruptly end after 14 residues (10).

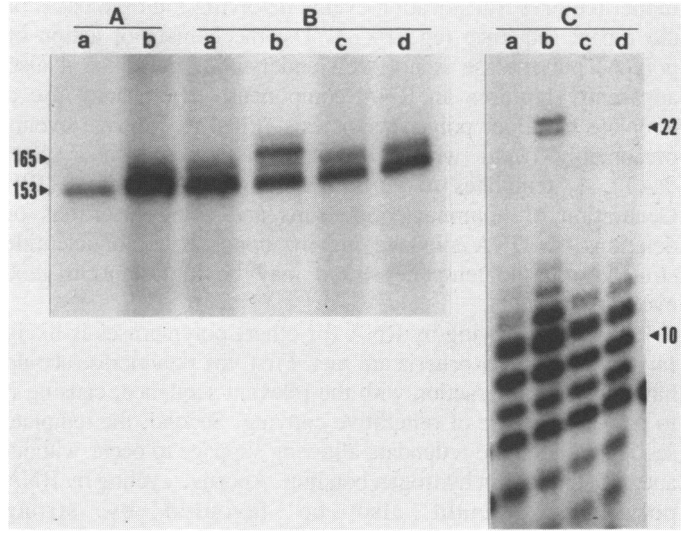

Figure 4. Transcription of pTA22 and pTA33 *tet* gene fragments in mixed reactions. Full length run-off transcripts **(A,B)** and RNase T1 digestion products are shown **(C)**. **(A)** The pTA22 template (0.3 pmol) was incubated in the standard transcription reaction for 5 min **(a)** or 30 min **(b)**. **(B,C)** At 5 min, pTA33 fragment (0.3 pmol) was added to a portion of the reaction **(b,d)** simultaneously with excess (2.5 mM final concentration) UTP **(a,b)** or ATP **(c,d)** and the reaction was continued for an additional 25 min. Numbers correspond to size of RNA products in nucleotides.

Survey of transcription start points

We have recently compiled and analyzed 263 *E. coli* promoters for which there is experimental data on the site of transcription initiation (2). The generality of the anomalous behaviour of RNA polymerase reported for pTA22 and pTA33 promoters is difficult to determine from this analysis since common techniques used for mapping the 5' end of transcripts, such as sizing RNA, genetic analysis, and S1 nuclease mapping, do not always pinpoint the exact transcription start site or allow anomalous 5' ends to be detected. Nevertheless, heterogeneity in 5' ends was often reported (2), and in most cases this was not believed to result

from technical limitations. Therefore, the compilation can be used to test the prediction that slippage, and hence heterogeneity, occurs more frequently when transcription initiates opposite homonucleotide stretches on the template strand. Thirty-three of the 263 promoters analyzed have initiation points which lie within stretches of 3 or more identical bases. Of these, 66% have multiple start points reported. The frequency of multiple starts reported for other promoters (ones in which the initiation site is not within a homonucleotide stretch) is only 14%. Although these data cannot be used to identify reiterative copying, they support the slippage model. Additional studies are required to establish the generality of reiterative copying.

Significance

Synthesis of oligo(A) by RNA polymerase was observed in the early 1960s (4,5) in *in vitro* reactions with artificial templates and ATP as the sole templated ribonucleotide precursor. It was concluded that RNA polymerase slipped during elongation opposite homonucleotide (T) stretches in the template strand, but that this was not physiologically relevant since *in vivo* templates are double stranded and all four nucleotide precursors are present. Our studies, and others demonstrating reiterative copying by RNA polymerase using DNA fragments containing natural promoters (3,7–10) suggest that this process may occur *in vivo*, if not in transcription, then perhaps in other systems where specific chain extension is catalyzed with limited template. For example, the telomere transferase ribonucleoprotein complex extends ends of linear DNA fragments with repetitive sequences. Greider and Blackburn (17) have suggested that telomerase utilizes a complementary portion of the RNA component of the complex as a template for reiterative copying. Telomere extension by this model requires translocation events involving multiple bases of the repeat sequence (eg. T_2G_4). The mechanism of action of poly(A) polymerase is not well understood, but since it also apparently requires an RNA component (18), it may use a template to direct poly(A) synthesis. Finally, slippage events presumably occur when DNA polymerase replicates short $d(AT)_{8-14}$ templates *in vitro*, generating poly($d(AT)_n$) (19). Generation of homonucleotide runs and small insertions or deletions by DNA slippage *in vivo* opposite homonucleotide stretches on the template strand may be important in gene evolution (20,21).

Reiterative copying by RNA (or other) polymerases is likely favoured when two criteria are met. First, the polymerase should have a strong interaction with the relevant sequence, causing it to pause at the site of reiterative copying. Second, the template sequence should be redundant allowing slippage to occur without a significant loss in hydrogen bonding. Abortive cycling by RNA polymerase should also be favoured by strong polymerase/promoter interactions (22), which is sometimes (23), but not always observed (10,16). The mechanism and significance of reiterative copying by polymerases warrants further investigation.

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