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Mutations affecting primer RNA interaction with the replication repressor RNA I in plasmid ColE1: potential RNA folding pathway mutants

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The control of plasmid ColE1 copy number is mediated by the kinetics of interaction of two complementary plasmid-encoded RNAs. One RNA is the primer precursor and the other is a small counter-transcript called RNA I. The interaction of these highly structured RNAs results in inhibition of formation of mature primer RNA necessary for replication initiation. We have studied several plasmid copy number mutants which have single base changes in the primer which render the primer resistant to inhibition by RNA I despite the fact that the mutations are located outside the overlap between primer and RNA I. We propose a model to account for the resistance of the mutant primers which is based on the differential folding of the nascent primer transcripts during transcription. We propose that the mutant primers diverge in structure from their wild-type counterparts during a discrete period during transcription. During this brief divergence, they are proposed to interact kinetically more slowly with RNA I than wild-type primer because a particular domain (the anti-tail) required for efficient interaction with RNA I is buried in a stem-loop structure while this same domain is predicted to be single-stranded in the wild-type. Despite substantial sequence divergence from ColE1, the primer precursors of the related plasmids CloDF13, RSF1030 and p15A also have retained the potential to expose their anti-tail in a similar manner to ColE1, suggesting that the folding pathway has been conserved in evolution. We show that the RNA polymerase pause pattern during primer transcription of the mutants is locally different from that of wild-type primer, consistent with the idea that they have different conformations. The model makes predictions about the copy number phenotype of new mutants at the same position as those we have isolated. To test the model we constructed site-specific mutations and determined that their copy number phenotype *in vivo* was consistent with the predictions of the folding model. These results suggest that transient conformational features of an RNA molecule during transcription can play a key role in determining its functional fate.

Key words: ColE1/copy number/replication initiation/RNA interaction

Introduction

The control of replication initiation of the *Escherichia coli* plasmid ColE1 serves as a model system for understanding how a stably inherited replicon is maintained at a steady-state copy number (for reviews, see Polisky, 1988; Simons and Kleckner, 1988). Central to the regulation is the interaction between two complementary plasmid-encoded RNA molecules. One of these RNAs is transcribed initially as a precursor to the mature primer RNA for leading DNA strand synthesis (Itoh and Tomizawa, 1980). The other RNA, known as RNA I, is complementary to the 5'-terminal region of the primer precursor. RNA I plays a negative role in the regulatory circuit as a consequence of its interaction with the primer precursor (Tomizawa and Itoh, 1981; Lacatena and Cesareni, 1981). In the absence of RNA I, the primer precursor RNA has the unusual ability to form a stable RNA-DNA hybrid with the DNA template strand in the vicinity of the replication origin (Itoh and Tomizawa, 1980). The ability of primer to form a hybrid is a sensitive function of RNA structure since mutations altering structure have been isolated which are defective in hybrid formation and replication defective *in vivo* (Masukata and Tomizawa, 1984). The RNA-DNA hybrid can be recognized by ribonuclease H which cleaves the RNA moiety of the hybrid, generating a properly positioned 3'-OH residue which serves as a primer for the addition of deoxyribonucleotides by DNA polymerase I. If primer precursor transcription occurs in the presence of RNA I, formation of the primer precursor RNA-DNA hybrid is reduced; leading DNA strand synthesis is consequently reduced because DNA polymerase I inefficiently recognizes unprocessed primer precursor (Tomizawa and Itoh, 1981).

RNA I inhibits primer precursor processing as a result of its direct interaction with the complementary RNA. Both RNAs have highly ordered structures. RNA I is 108 nucleotides in length and consists of four well defined structural domains: three stem-loop domains and a 5'-terminal single-stranded region (Morita and Oka, 1979; Tamm and Polisky, 1983; Masukata and Tomizawa, 1986). The interaction between the complementary RNAs alters secondary and tertiary conformational aspects of the primer RNA which are required to permit it to form an RNA-DNA hybrid at the origin (Masukata and Tomizawa, 1986). The details of the interaction have been studied by both biochemical and genetic manipulations. In the initial stages of the interaction, the complementary single-stranded loop domains 'kiss' via formation of Watson-Crick hydrogen bonds (Tomizawa, 1984). Subsequently, the 5'-terminal region of RNA I nucleates hybrid formation with its complement in the primer precursor. The interaction ultimately results in a full length RNA-RNA duplex (Tomizawa, 1985; Tamm and Polisky, 1985).

Kinetic considerations play a key role in the inhibition of primer processing by RNA I. RNA I is only effective as an inhibitor if it intercepts primer during a particular window during primer transcription (Masukata and Tomizawa, 1986). If RNA I encounters primer after this window, it may still interact with primer but the interaction is without

functional consequence. These observations suggest that primer undergoes dynamic structure alterations during its synthesis which modulate its sensitivity to the counter-transcript inhibitor. Biochemical evidence for a particular conformational reorganization during primer transcription involving the first 200 nucleotides has been obtained (Wong and Polisky, 1985); this rearrangement has been correlated with the ability of the nascent primer to be properly regulated by RNA I (Masukata and Tomizawa, 1986).

We have been studying the molecular basis for the high copy number behavior of a set of mutations in the ColE1 plasmid. These mutations caused plasmid copy number to increase dramatically under certain cell growth conditions, such as entry into stationary phase or after a temperature shift (Fitzwater *et al.*, 1988). These mutations map to a particular primer domain which lies outside its overlap with RNA I (Figure 1). The plasmid DNA amplification observed *in vivo* occurs in the presence of wild-type RNA I repressor encoded by the mutants. For one of the mutations, called pMM7, we showed that mutant primer RNA processing by RNase H was resistant to inhibition by purified RNA I *in vitro*, under conditions where the wild-type primer processing was sensitive (Fitzwater *et al.*, 1988). These observations indicated that the molecular basis for the DNA amplification was likely to be resistance of the mutant primer to inhibition by RNA I despite the fact that both components were wild-type in their complementary sequences.

In this report, we propose a molecular model for the resistance of each of the mutant primers to inhibition by RNA I. The model proposes that each mutation similarly but not identically alters the dynamic folding pathway of nascent primer during a brief temporal window in a way that transiently alters its structure relative to wild-type primer. The novel conformations assumed by mutant primer are predicted to reduce their sensitivity to RNA I inhibition compared to wild-type primer of identical chain length. According to the model, before and after this window, mutant primer structure and sensitivity to RNA I are identical. We have obtained biochemical evidence that the mutations indeed affect primer conformation by showing that each mutant specifically alters the pattern of transcriptional pausing of primer during a brief period when the mutations themselves are being transcribed. Finally, we tested the ability of the model to predict copy number phenotype by creating site-specific mutations designed to change the structure of mutant primer. These new mutants have the copy number phenotype *in vivo* predicted by the model.

Results

Mutant primer RNA processing is resistant to inhibition by RNA I

The copy number mutations studied here have been described previously (Fitzwater *et al.*, 1988); the sequence changes responsible for their phenotypes are shown in Figure 1. Three of the mutations are single base changes at primer positions +128 (pMM7), +131 (pMM4) and +132 (pMM1). One mutant plasmid (pEW2705) has two altered bases at positions +124 and +134. All of these mutations alter non-conserved elements of the RNA I promoter but do not significantly affect the strength of this promoter *in vitro* or *in vivo* (Wong *et al.*, 1982; Fitzwater *et al.*, 1988); these and other data indicate that these mutants do not affect RNA I synthesis or stability *in vivo*. The observation that these plasmid mutants have similar (though not identical) copy number behavior, and their clustered location led us to look for a common model that might account for their properties.

We have shown previously that the primer RNA made by one of the mutants, pMM7, is resistant to RNA I inhibition *in vitro*. To investigate whether this was true for the other mutants in this region, we measured RNase H sensitivity of primer transcribed in the presence and absence of wild-type RNA I. In these experiments, primer RNA is specifically initiated and labeled in its 5′-terminal region with [α-^{32}P]ATP. Two classes of transcripts are produced in the absence of RNase H (Masukata and Tomizawa, 1984). One is a discrete set of transcripts hybridized to the template DNA strand. These can be cleaved to the mature primer size of ~555 nucleotides. The other set consists of larger, heterogeneous sized transcripts that have not hybridized to the origin region and cannot be processed. The effect of RNA I addition (2.2×10^{-8} M) on the processing of wild-type and mutant primer precursors is shown in Figure 2. In the presence of RNase H, the wild-type hybridized primer precursors are converted to the mature 555 nucleotide primer (Figure 2, lane 2). The addition of RNA I to the wild-type transcription reaction blocks RNase H cleavage by preventing hybridization of wild-type primer to the template strand (Figure 2, lane 4). Quantitation of the 555 nucleotide region in this lane indicated that wild-type mature primer formation was inhibited by 95% under these conditions. Compared to the wild-type primer, each mutant primer is resistant to inhibition of RNase H processing by RNA I; a prominent 555 nucleotide product appears in each mutant transcription reaction (Figure 2, lanes 6, 8, 10, 12). Similar resistance of mutant primers to RNA I inhibition was also seen at 4-fold higher RNA I concentrations (data not shown). We conclude from these experiments that each of the four mutant primers is resistant to RNA I inhibition *in vitro*.

Possible mechanisms to account for RNA I resistance

Two potentially inter-related hypotheses can be made to account for the resistance of the mutant primers to RNA I. Perhaps the more obvious idea is to postulate that the mutations change the conformation of the primers such that they do not interact with RNA I with proper kinetics, or interact in a non-functional manner. This type of model has been useful to explain the behavior of certain copy number mutants of ColE1 which have single base changes in the primer−RNA I overlap region; although RNA I and primer are fully complementary in these mutants, the structural changes caused by the mutations lead to decreased kinetics of interaction of homologous mutant RNA components (Tomizawa and Itoh, 1981). In the case of the mutants here, however, both RNA I and the region of the primer complementary to RNA I are fully wild-type. Thus, during the early stages of primer formation, the interaction between mutant primer precursor RNA and RNA I must be identical to that occurring in the wild-type plasmid.

A second possibility to explain the mutants is to postulate that the conformational changes introduced by the mutants do not directly affect kinetics of interaction with RNA I, but rather alter the extent of time spent in various nascent conformations by the transcribing RNA polymerase. By altering the pausing pattern of RNA polymerase during its transit of the primer gene for example, the mutations could increase the time nascent primer appeared in conformations

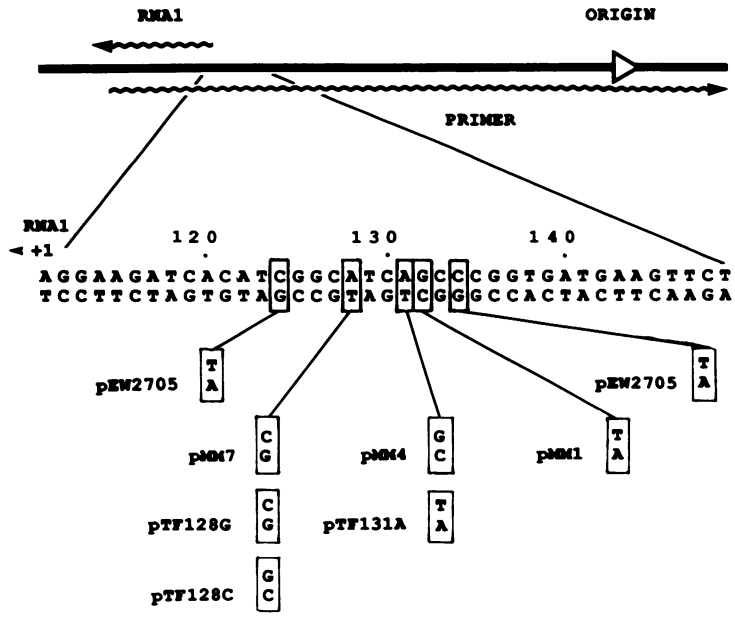

Fig. 1. Location of plasmid copy number mutations. The upper thick line represents a part of the ColE1 plasmid genome. The two wavy lines represent the primer transcript and the RNA I transcript. The open triangle represents the origin of leading DNA strand synthesis. Shown below is a portion of the DNA sequence encoding the primer RNA upstream of the RNA I transcription start. The numbers above the sequence refer to positions in the primer transcript. Basepairs in rectangles denote mutant sites. The construction of pTF128G, pTF128C and pTF131A are described in Materials and methods.

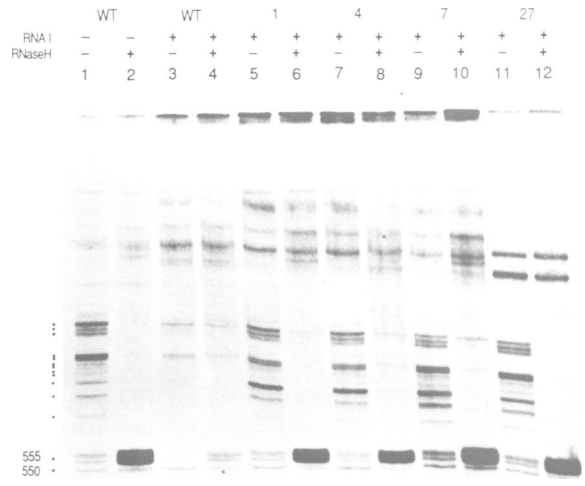

Fig. 2. Resistance of mutant primer RNA processing to inhibition by RNA I *in vitro*. Supercoiled plasmid DNAs were transcribed by *E.coli* RNA polymerase as described in Materials and methods. Primer RNA was labeled in the 5′-terminal region with [^{32}P]ATP. A single round of primer transcription was carried out by addition of rifampicin to block subsequent initiation. Transcription was carried out in the presence and absence of purified ColE1 RNA I, and the presence and absence of RNase H. Transcription and processing of mutant primer RNAs synthesized in the absence of RNA I are not shown, but their electrophoretic patterns were identical to those carried out in the presence of RNA I. Primer transcription reactions were electrophoresed on a 6% polyacrylamide gel containing 8 M urea, dried and autoradiographed. Equal numbers of counts were loaded in each lane. The figure shows the autoradiogram. RNA I was prepared and purified from separate transcription experiments *in vitro* as described in Materials and methods. Designations at the top of the figure refer to the template DNA used to transcribe primer. WT refers to the wild-type plasmid, pNOP42; 1, 4, 7, 27 to pMM1, pMM4, pMM7 and pEW2705 respectively. The numbers at the side are nucleotide size markers. Dots along the side of the leftmost lane denote the mobility of transcripts that are the hybridized primers which are substrates for RNase H. Upper bands are non-hybridized transcripts.

unfavorable for RNA I interaction, or decrease the time spent in favorable conformations.

We have examined the first possibility experimentally in its simplest form by attempting to determine whether wild-type and mutant primer RNAs can be distinguished by either their secondary structure or their kinetics of interaction with RNA I *in vitro*. The kinetics of interaction of primer RNA and RNA I can be quantitated *in vitro* by measuring the rate with which they form an RNA−RNA duplex (Tomizawa, 1984; Tamm and Polisky, 1985). As a labeled component enters duplex form its mobility shifts upon electrophoresis on a non-denaturing acrylamide gel compared to 'single-stranded' RNA. Using this assay we have previously compared the rates of association of RNA I with a purified primer run-off transcript of 241 nucleotides (p241) made either from wild-type or pMM7 templates. We were unable to detect any difference in the kinetics of association of RNA I with these primers (Fitzwater *et al.*, 1988). In addition, when we compared secondary structural features of these p241 species by limited nuclease digestion *in vitro*, we were unable to detect any conformational differences between them (results not shown). We conclude that by the time primer RNA is 241 nucleotides in length, there is no apparent conformational or functional difference between mutant and wild-type primer.

As RNA is synthesized, it is plausible to assume that it exists in a variety of transient conformations as elongation proceeds (Kramer and Mills, 1981). Downstream palindromic domains can disrupt and reorganize upstream stem−loop domains. The ColE1 primer itself provides an example of this phenomenon. During its transcription *in vitro*, a major structural rearrangement has been observed between primer positions +73 and +197 in which an initially formed stem−loop domain (called stem−loop III) is restructured, ultimately resulting in the formation of a larger hairpin domain called stem−loop IV (Wong and Polisky, 1985). This reorganization in conformation has an important effect on primer fate; hybridization of primer with

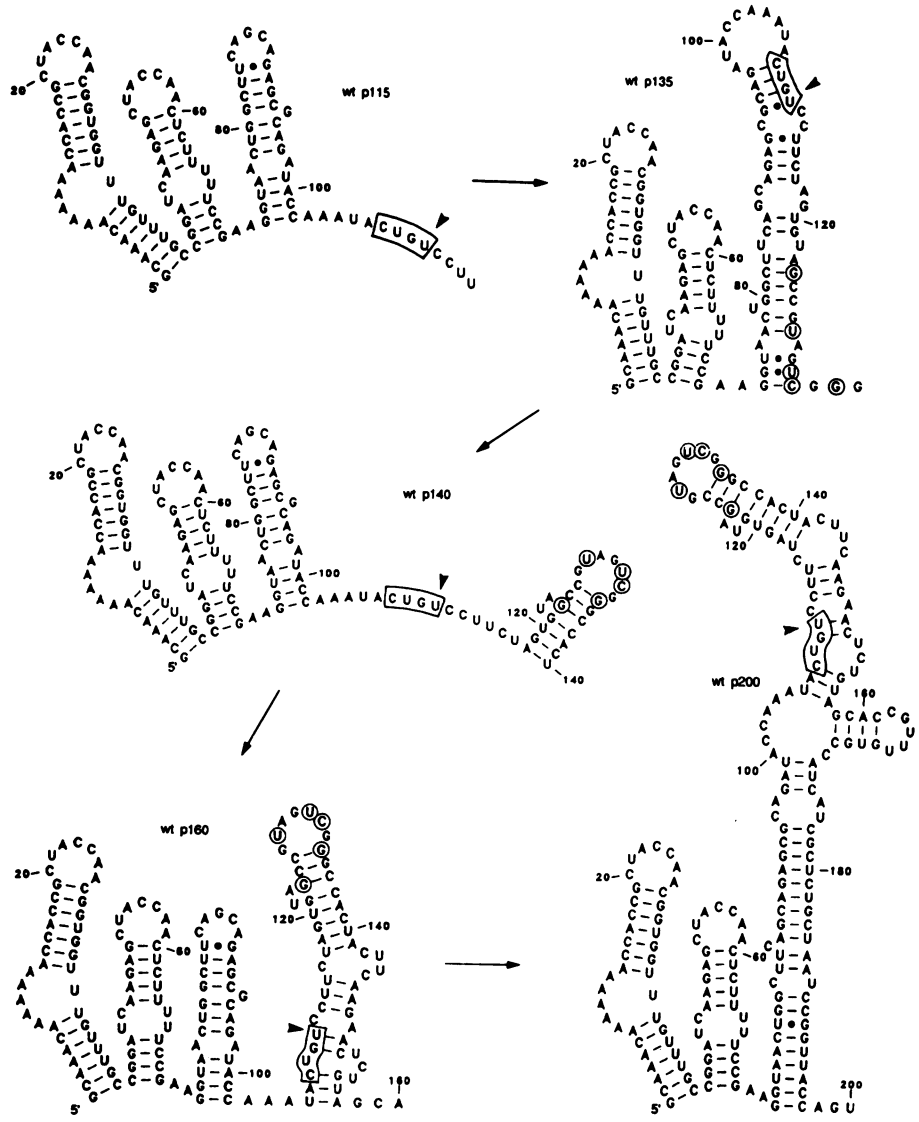

Fig. 3. A potential dynamic folding sequence for nascent wild-type ColE1 primer RNA. Selected lengths of primer RNA were folded using the FOLD program (Zuke and Steigler, 1981) using the energy parameters of Freier *et al.* (1986). The lengths were chosen to best illustrate the potential alteration of structures of regions thought to be important for primer–RNA I interaction as primer elongates. Additional intermediate structures are predicted to form at elongation positions intermediate to the positions shown. These have been omitted for clarity. Minimal free energy secondary structures are shown. The bases enclosed by a rectangle represent the 'anti-tail', i.e. sequences complementary to the single-stranded tail of RNA I. The arrowhead denotes the position complementary to the 5′-terminal nucleotide of RNA I. The circle bases are those positions altered by the mutations studied here. Note that when primer is 200 nucleotides, all mutant positions except EW2705 are present in a loop. This domain is not predicted to be altered by subsequent primer elongation.

the DNA template strand in the origin region is correlated with ability of the primer to form stem–loop IV (Masukata and Tomizawa, 1986). Although the exact mechanism by which formation of stem–loop IV affects primer is unknown, these results have suggested that an important decision is made in this temporal window of primer formation.

The 'anti-tail' flicker model

We undertook to test the idea of dynamic conformational changes in nascent transcripts to understand the behavior of the mutants. We considered the possibility that they affected primer secondary structure only during a brief period after they appeared in the elongating primer chain. To assess the plausibility of this idea, we used a computer folding program that provides minimum free energy structures for RNA (Zuker and Steigler, 1981). To simulate the transcription process, we folded wild-type and mutant primers of systematically increasing length and compared the secondary structures that were predicted to progressively form. Because these folding programs are most useful for predicting local conformations, we confined our attention to primer structure in the vicinity of the mutations. We were particularly interested in the various conformations assumed by primer sequences complementary to the three loops and 5′ single-stranded tail of RNA I because these structural features are known to be important to optimal kinetics of RNA–RNA interaction (Tomizawa, 1984; Tamm and Polisky, 1985).

Schematic diagrams of the predicted secondary structures of nascent RNA chains of 115, 135, 140, 160 and 200 nucleotides for wild-type are shown in Figure 3. A similar analysis was carried out with each mutant primer. This

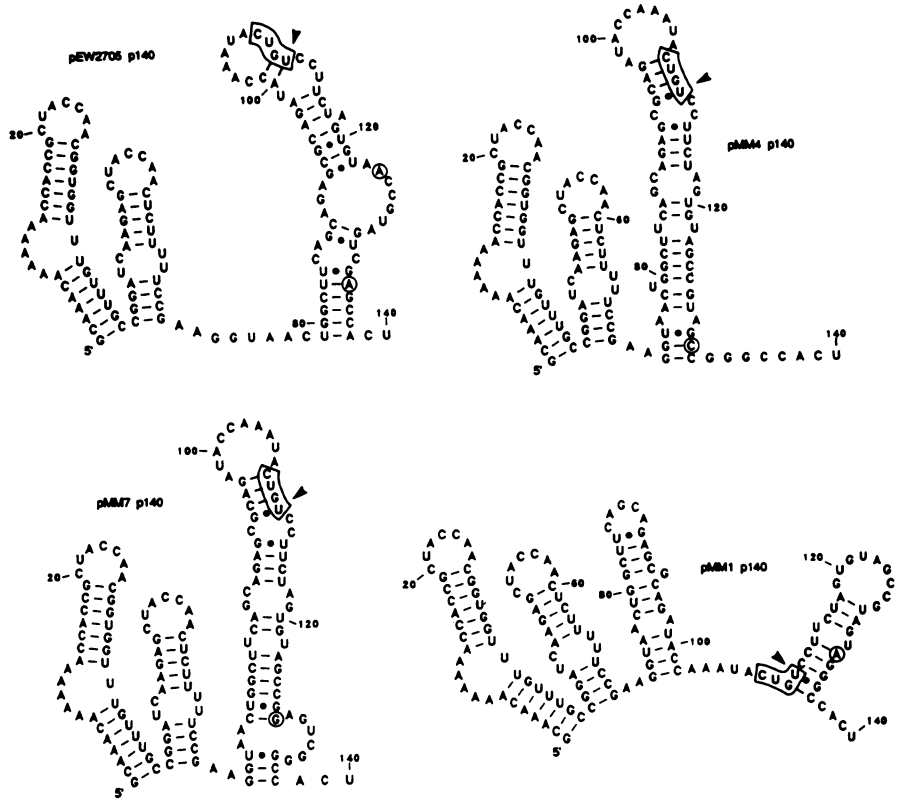

Fig. 4. Minimal free energy secondary conformation for mutant primers of 140 nucleotides. The four mutant primers each contain a single base difference from wild-type, except for pEW2705, which contains two changes. The bases altered by mutation are circled. The anti-tail is enclosed in a rectangle. The arrowhead represents the position complementary to the 5′-terminal nucleotide of RNA I. These structures should be compared to wild-type primer 140 in Figure 3.

analysis provided support for our initial hypothesis of transient conformational differences between wild-type primer and all the mutants. The computer folding program predicted that at short (115) and longer (200) lengths, mutant and wild-type primers had identical conformations. However, they differ at intermediate lengths. In particular, mutant and wild-type primer conformations differ in the region of 140 nucleotides, and the difference is provocative (Figure 4). In p140wt, primer RNA has three stem−loops similar to p115wt, and the region complementary to the single-stranded tail of RNA I (the 'anti-tail') is in an apparently accessible, single-stranded conformation (see wt p140, Figure 3). We describe the predicted reappearance of the anti-tail in wild-type primer as the 'anti-tail flicker' because after the addition of four additional bases to elongating primer the anti-tail is predicted to be reorganized into a new stem domain.

In contrast to the wild-type primer, p140MM7 is folded into a three stem−loop conformation in which the anti-tail is present in a stem, and less accessible for interaction with the RNA I tail (Figure 4). Also, one of the potential loop−loop interactions between the RNAs is absent in p140MM7. When progressive folding of other copy number mutant primers was carried out, they showed similar, but not identical conformational differences from wild-type primer (Figure 4). In each case, the mutant primers were predicted to transiently assume a conformation in which the anti-tail was buried in a stem structure and a potential loop−loop interaction with RNA I was missing. The basis for the conformational difference between wild-type and mutant primers was the potential in each case to stabilize a new stem−loop domain downstream of the mutation by formation of a Watson−Crick base pair involving the mutant base. For example, the U to G change in pMM7 permits a G128−C78 pair in nascent primer of 140 nucleotides (see Figure 4). Similarly, the U to C change in pMM4 permits a C131−G73 pair in the mutant primer p140. Each mutant primer could assume a conformation similar to the wild-type up to approximately position 135, but significantly diverged by being unable to assume a conformation like that of wild-type at position 140. These considerations suggested a basis for the altered susceptibility of each mutant primer to RNA I *in vitro*, viz. an altered conformation which interacted with reduced kinetics with RNA I.

In summary, wild-type primer is predicted to undergo a dynamic 'anti-tail flicker' in a transcription window between positions 140 and 144. The copy number mutants either eliminate the potential for this change or alter its lifetime.

The potential to anti-tail flicker is conserved in evolution

One approach to assess the plausibility of the folding hypothesis is to make a phylogenetic survey of plasmids related to ColE1. ColE1 is related to, but compatible with, other members of a multicopy plasmid family including plasmids such as CloDF13, RSF1030 and p15A. These plasmids have similar copy number control mechanisms to ColE1; each encodes an incompatibility specific RNA I species that interacts preferentially with homologous primer precursor RNA. Both RNAs from each plasmid differ from

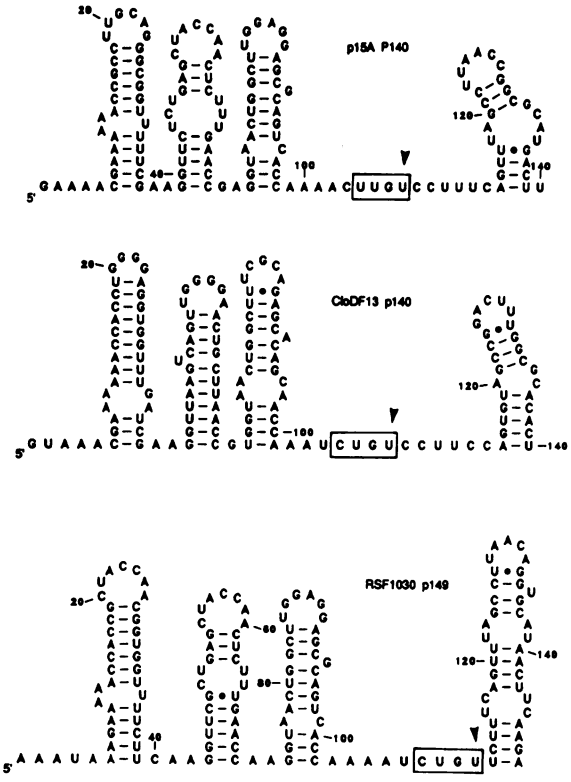

Fig. 5. Minimal free energy secondary stuctures of the ColE1-related plasmids p15A, CloDF13, and RSF1030. The rectangle and arrowhead are as in previous figures. Sequences are from Selzer *et al.* (1983). Each of these structures is a transient intermediate derived in a folding pathway similar, but not identical to that of ColE1. These structures should be compared to the ColE1 p140 structure in Figure 3.

their ColE1 cognate in primary sequence but nonetheless can be folded into secondary structural domains similar to those of ColE1 (Selzer *et al.*, 1983). The extent of sequence divergence is illustrated by the p15A primer which differs from ColE1 in 44 of the first 140 nucleotides; the RSF1030 primer differs at 41 positions, and the CloDF13 primer at 43 positions. To determine whether the potential anti-tail flicker conformation might be conserved despite these sequence changes, we carried out a progressive folding analysis on each of these primers. All three primers show the potential for the anti-tail flicker (Figure 5); p15A primer at positions 139–140, RSF1030 at positions 149–150 and CloDF13 at positions 139–145. Thus, the possibility of assuming the 'anti-tail flicker' conformation is evolutionarily conserved despite extensive primary sequence changes.

The mutations alter the transcriptional pause pattern of primer RNA

Obtaining physical evidence for the transient conformational alterations predicted by the model is a formidable technical problem. As mentioned earlier, in addition to their possible effects on the kinetics of RNA–RNA interaction, alterations in nascent primer conformation might be manifested in changes in the pausing pattern of RNA polymerase during primer transcription. To investigate this possibility, we compared the transcriptional pause pattern of wild-type and mutant primer RNAs *in vitro* (Figure 6). In these experiments, a single round of transcription was specifically and synchronously initiated at the primer promoter on supercoiled plasmid DNA templates (see Materials and methods). Samples of nascent primer RNA, radiolabeled at the 5-terminal region, were removed at various times and electrophoresed. In addition to the four mutants pMM7,

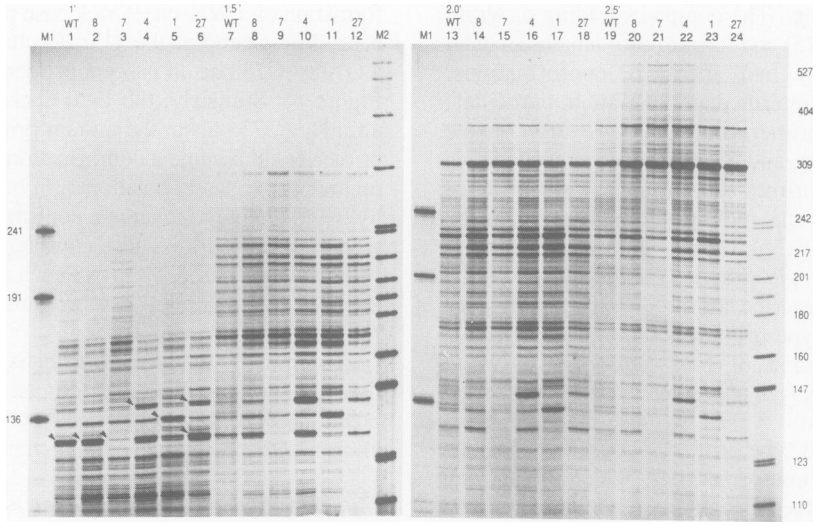

Fig. 6. Transcription pause pattern during synthesis of wild-type and mutant primer RNAs *in vitro*. Transcription was selectively initiated at the primer promoters of wild-type and mutant supercoiled DNA templates using *E.coli* RNA polymerase as described in Materials and methods. Primer RNA was labeled at its 5'-terminal region with [^{32}P]ATP. Elongation of primer transcription was stopped at 1.0, 1.5, 2.0 and 2.5 min after elongation of the initially synthesized 21 nucleotide primer fragment (see Materials and methods). The labeled primers were purified by phenol extraction and ethanol precipitated, then electrophoresed on a 6% polyacrylamide gel containing 8 M urea and autoradiographed. The samples on the autoradiogram are organized by time to facilitate comparison of the nascent transcript size distribution. **Lanes 1–6**, 1 min; **7–12**, 1.5 min; **13–18**, 2.0 min; **19–24**, 2.5 min. The DNA templates used for the transcription are shown at the top of each lane. WT, pNOP42; 8, pMM8; 7, pMM7; 4, pMM4; 1, pMM1; 27, pEW2705. Lanes M1 are purified primer RNA size markers made by run-off transcription of restriction-cleaved pNOP42 DNA; their sizes are shown to the left of the figure. The M2 lane shows denatured DNA size markers of lengths shown at the right of the figure. Arrowheads in **lanes 1–6** denote transcripts whose appearance is affected by mutation.

pMM4, pMM1 and pEW2705, which all occur in a localized region, we also transcribed another high copy number mutant called pMM8 (Muesing *et al.*, 1981). pMM8 contains a G-C to A-T translation mutation in the RNA I−primer overlap, at position +53 of primer (corresponding to position +58 of RNA I. Its high copy number phenotype is a consequence of a defective RNA I−primer interaction resulting from alteration of RNA I conformation (data not shown). pMM8 serves as an independent control for the transcriptional pausing pattern of the other high copy number mutants since its pause pattern was not expected to differ from that of wild-type primer.

Figure 6 shows the distribution of pause sites in primer RNA from wild-type and the five mutant DNA templates at various times after the initiation of transcription. It is apparent that RNA polymerase pauses at a number of positions during transcription of wild-type primer RNA (lanes 1, 7, 13, 19). As expected, the details of the pause pattern are essentially identical between wild-type primer (pNOP42) and the copy number mutant pMM8 (compare lanes 1 and 2). However, inspection of the other mutants indicates that each of them has a distinctive pause pattern during the early phases of primer transcription. For example, RNA polymerase does not pause at two adjacent positions in the pMM7 primer where pausing is prominent in the wild-type primer (positions +127, +128; compare lanes 1 and 3, Figure 6). Note that the mutation in pMM7 DNA is at position +128, coincident with the more prominent pause site. In contrast to pMM7, the pause patterns of pMM4 and pMM1 show enhancement at sites that are momentary pauses for the wild-type primer (compare lanes 1 and 4, 5). These enhancements are at position +139 for pMM4, and +133 for pMM1 (compare lanes 1 and 6 with lane 3, Figure 6). The pause pattern for pEW2705 is only slightly altered from that of wild-type, but is qualitatively similar to that of pMM4, showing enhancement at +139 (compare lanes 1 and 6, Figure 6). These patterns were unaffected by the addition of saturating amounts of purified NusA protein, which is known to affect pausing at certain sites in other transcripts (Kassavetis and Chamberlin, 1981; data not shown).

The pause patterns of mutant and wild-type primers are identical once RNA polymerase proceeds beyond the vicinity of the mutants (lanes 7−24, Figure 6). Thus, to the extent that the pause pattern reflects local RNA conformation, these results indicate that, as predicted by the conformational folding model, the mutant primers differ in conformation from wild-type during a brief temporal window during and briefly after the synthesis of the region of primer containing the sequence changes. These changes appear not to have global conformational consequences.

Testing the model with site-directed mutants

If the anti-tail flicker model is the actual basis for the phenotype of the mutants described here, it should be possible to use the model to predict the phenotype of new mutants in the same region of the plasmid genome. In particular, we assessed whether an alternative base at the position of known mutants would affect the ability of the primer to anti-tail flicker. For example, the mutant pMM7 has a G at position 128 instead of the wild-type U, permitting formation of the G128−C78 interaction presumed to prevent the anti-tail flicker (see Figure 4). However, substitution of a C at position 128 is predicted to preclude this interaction; primer carrying this change shows anti-tail flickering indistinguishable from wild-type when subjected to the progressive folding analysis (result not shown). Similarly, changing primer position 131 from the wild-type U to A is predicted to have no effect on primer folding in contrast to pMM4 which has a G at this position (see Figure 4).

ColE1 derivative plasmids containing either the p128C or p131A mutations were created by site-specific mutagenesis as described in Materials and methods. The mutants were generated from wild-type DNA. Plasmids containing these changes were readily identified because they create new restriction sites. Their copy number phenotype was determined qualitatively in *E.coli* strain DG75 carrying a compatible reference plasmid (pTF487). In addition to the two new mutants we also recreated the base change present in pMM7 (128G replacing the wild-type U) to serve as a positive control for mutant copy number phenotype. The copy number phenotypes of these mutants were compared after growth into stationary phase at 37°C. Cleared lysate samples from equal numbers of cells were electrophoresed on an agarose gel. In contrast to the phenotype of a derivative carrying the p128G alteration, the copy number phenotypes of the p128C and p131A mutants were observed to be indistinguishable from wild-type (Figure 7A). These results correlate with the predictions of the anti-tail flicker model.

As mentioned previously, the mutant plasmids lack the *rop/rom* gene. We tested the effects of providing the *rop/rom* gene product in *trans* on the copy number amplification displayed by pMM7 and pTF128G by using the compatible plasmid pGC8, which carries the pBR322 *rop/rom* gene. As shown in Figure 7B, the Rop/Rom protein suppresses the amplification of these plasmids, and, as expected, lowers the copy number of the wild-type plasmids and the site-directed mutants pTF128C and pTF131A.

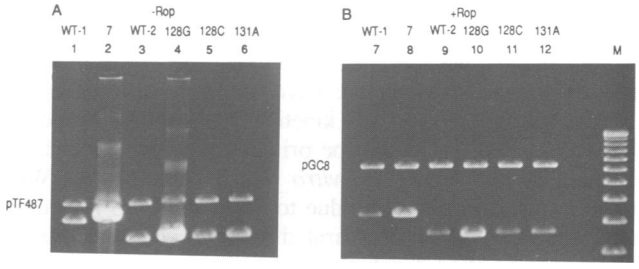

Fig. 7. Copy number behavior of wild-type and mutant plasmids in the presence and absence of the *rop* gene *in vivo*. Cleared lysates were prepared from late stationary cultures containing either wild-type or mutant plasmids. In addition to the ColE1 plasmid, each culture carried either the compatible rop^+ plasmid pGC8, or the rop^- GC8-derivative plasmid pTF487. The pairs of plasmids in identical numbers of cells were linearized by digestion with *Pst*I, electrophoresed on a 0.8% agarose gel, stained with ethidium bromide and photographed. The compatible plasmids serve as copy number and load controls. **A**, cells carried the rop^- plasmid, pTF487; **B**, cells carried the rop^+ plasmid, pGC8. Linear pTG487 and pGC8 are the upper bands in **A** and **B** respectively. The linearized ColE1 derivative plasmids are the lower bands in each lane. Designations at the top of the lanes refer to the ColE1 derivative plasmid present in the cells. WT-1, pNOP42, the 4.2 kb wild-type parent; 7, pMM7; WT-2, pSTK131, the 3.6 kb wild-type parent of the site-directed mutants; 128G, pTF128G containing a G at primer position 128; 128C, pTF128C containing a C at position 128; 131A, pTF131A containing an A at position 131. M is a 1018 bp DNA ladder; the lowest band in the lane is 3045 bp. The higher mol. wt bands in **lanes 2** and **4** in **A** are uncleaved multimeric forms of pMM7 and pTF128G respectively.

Discussion

In this paper we have studied several unusual copy number mutants of colE1 which display a high copy number phenotype, yet encode a wild-type RNA I repressor. In a purified transcription system *in vitro*, we have shown that the primer precursor encoded by the mutants is resistant to the inhibitory action of RNA I. We have developed a model to account for the resistance of the mutant primer RNA which is based on an altered conformational pathway assumed by the nascent mutant primer RNA chain during its elongation compared to that of the wild-type primer. The model predicts that certain mutant intermediates will interact more slowly with RNA I than their wild-type counterparts due to an altered conformation of critical domains required for optimal interaction. We have obtained evidence consistent with localized conformational differences between mutant and wild-type nascent primers. In addition, we showed that the model correctly predicts the copy number behavior of several new mutants. The model focuses attention on an early phase of primer transcription as being critical for primer sensitivity to RNA I *in vivo*. Our results suggest that the critical window may be confined to a segment of primer transcription in the vicinity of 140 nucleotides.

In principle, sequential folding analysis permits assignment of the positions where the window opens and closes. For example, pMM7 and wild-type primer are predicted to diverge slightly in conformation at position +130, i.e. shortly after transcription of the mutation site, and merge to identical conformations after position +151. pEW2705 primer is somewhat different. It is predicted to diverge quite radically by position +130 and remains distinct from wild-type primer even at position +200. Verification of these positions of divergence requires detailed structural information about the nascent primer conformation in the ternary complex of RNA polymeras, DNA and RNA. We have attempted to specify these parameters biochemically, i.e. by isolation and purification of nascent transcripts from wild-type and mutant templates after brief periods of *in vitro* transcription. However, we have not been successful in demonstrating differences in kinetics of association between isolated mutant and wild-type primers of length 135−150 nucleotides and RNA I *in vitro* (results not shown). We believe this failure may be due to the inability to renature important but subtle structural differences between these RNA species after denaturing gel isolation.

The observed changes in the transcriptional pause patterns between wild-type and mutant primer RNAs are generally interpreted to reflect local conformational differences in the nascent RNAs (with the exception of pMM7; see below). While this interpretation is plausible in light of the specific model we have proposed, the underlying causes of the changes are unclear, reflecting the fact that the events responsible for pausing remain unclear in general. While certain pauses have been correlated with nascent RNA hairpin formation *in vitro*, others are not (Levin and Chamberlin, 1987). The mutations differ substantially in their effects on pausing. The mutation in pMM7 (+128) is located at the site of a prominent primer pause site and greatly reduces pausing at this site. Pausing at the adjacent position (+127) is also reduced by the mutation. Thus, a template directed effect on pausing is possible for pMM7. However, the other three mutations enhance pauses at sites downstream from their positions, and these effects could be mediated by nascent RNA conformations. The pause patterns of wild-type and mutant primers do not provide explicit physical support for the anti-tail flicker model, e.g. we do not observe pause pattern differences in the 140−145 region that might reflect structures of the type postulated. Rather, the data support the idea of nascent conformational differences among the primers in the vicinity of the mutations.

This work emphasizes the functional importance of highly transient conformations in RNA and the subtlety of the RNA−RNA interaction that mediates ColE1 copy number control. It is likely that all RNAs undergo such fluctuations in structure during the transcription process (Kramer and Mills, 1981). In prokaryotes, where translation ensues coordinately with transcription, elongating ribosomes probably remove local stem−loop structures in mRNAs which might otherwise be stable. For untranslated RNAs, such as ribosomal or tRNAs, the ability to assume a compact, tightly folded conformation is assumed to be essential for proper interaction with protein ligands such as ribosomal proteins during ribosome assembly. The subtle interplay between the dynamics of transcription generating local RNA structure, and the progress of a translating ribosome is best illustrated in such fundamental control processes as transcriptional attenuation (Landick and Yanofsky, 1987). Transcriptional pausing and termination, each dependent on local RNA secondary structure, are the molecular events that are modulated during a brief temporal window during transcription; the decision to elongate transcription depends on the relative positions of a ribosome and RNA polymerase.

The ColE1 primer differs from these examples in several important ways. It is not translated, nor is it a stable structural RNA. During its synthesis a decision is made regarding its fate, viz. whether it is to hybridize to the template DNA strand and potentially act as a primer for leading DNA synthesis after processing by RNase H, or whether it is to be released from the DNA template. The decision is critically affected by the details of higher order primer conformation and by the association of the nascent primer with RNA I. The ability of RNA I to inhibit primer processing depends on its interaction during a critical period of primer transcription. This window has been investigated *in vitro* by Masukata and Tomizawa (1986), who have shown that nascent primer is sensitive to RNA I until ~360 nucleotides have been transcribed. After this point, RNA I is still capable of interacting with primer with rapid kinetics, but its binding does not affect subsequent hybridization. However, the critical conformational event that converts primer from a sensitive to resistant form is not known. The properties of the primer mutations described here suggest the possibility that under certain conditions *in vivo*, a critical step in primer folding occurs early in transcription, approximately between p130 and p170.

The progressive folding analysis used here to develop the model for copy number behavior is limited in several respects. First, the folding program provides information concerning the lowest energy structures using energy rules widely acknowledged to be incomplete (Turner *et al.*, 1988). Experimentation with the program reveals that many alternative conformations can be obtained which differ only marginally in free energy. Second, even if the predicted structures form, possibly kinetic barriers to progressive structural reorganization could potentially exist which could affect the length of time spent in a particular conformation by the growing RNA chain. A related concern is that less

stable structures might predominate kinetically during transcription. *In vivo* transcription rates are 30–50 nucleotides per second; in the absence of additional events such as pausing, this means that the time spent in a particular five nucleotide conformational window is on the order of 0.1–0.2 s. If RNA I is present at 100 copies per cell (2×10^{-7} M) and has an average K_a with primer of 1×10^6 M^{-1} s^{-1}, then the $t_{1/2}$ for the interaction is expected to be on the order of 120 s. This slow rate for complex formation compared to the fast rate of transcription may be reconciled by postulating that kissing, rather than complex duplex formation, is sufficient to alter primer conformation such that annealing to the template strand is prevented (Tomizawa, 1984).

In spite of these potential difficulties, on the basis of the following four observations we propose that the mutations reported here alter the dynamic conformation of nascent primer and consequently affect its functional interaction with RNA I: (i) each of the mutants is predicted to alter the conformation of a particular transient intermediate such that accessibility of the 'anti-tail' is reduced; (ii) compatible ColE1 relatives such as sp15A, RSF1030 and CloDF13 display the same potential intermediate as wild-type ColE1, suggesting evolutionary conservation of the folding pathway despite extensive sequence changes in primer; (iii) primer transcriptional pause analysis *in vitro* is consistent with each mutation perturbing local primer conformation in a distinct manner; (iv) site-directed mutations in primer predicted to be incapable of forming a conformation similar to that of p140mm7, in which the anti-tail domain is buried in a stem–loop domain, have wild-type copy number behavior.

The model can be tested further by attempting to suppress the copy number mutations with second site changes predicted to alter the bases contacted by the mutant base. Due to the overlapping nature of primer and RNA I, the second site changes must be made carefully so that RNA I function and stability are unaffected. Such tests are in progress.

The mutations described here have similar, though not identical, phenotypes. pMM1 and pEW2705 were originally isolated in searches for mutants with conditionally high copy number at 42°C, but normal copy number at 30°C. The other mutants, pMM7 and pMM4 arose from screens for elevated copy number at 37°C. We believe that the temperature effects on copy number reflect temperature-induced changes in nascent primer RNA conformation *in vivo*. The details of such changes relative to the model proposed here are not known.

We have shown that the Rop/Rom protein suppresses the amplification displayed by several of the mutants *in vivo* (Figure 7; Fitzwater *et al.*, 1988). The mutants differ in their sensitivity to the Rop/Rom protein *in vivo*. Unlike the other mutants, pEW2705 DNA contains the *rop/rom* gene, and amplifies in its presence. This protein catalyzes the reversible 'kissing' phase of the association of the complementary RNAs *in vitro* (Tomizawa and Som, 1984). The ability of the Rop/Rom protein to affect the interaction is highly sensitive to the conformations of the participating RNAs (Masukata and Tomizawa, 1986; Dooley and Polisky, 1987). Thus, the phenotypic suppression of the mutants *in vivo* by the *rop/rom* gene is consistent with the properties of the protein *in vitro*. If the mutant plasmid DNAs amplify as a result of the mechanism proposed here, then the Rop/Rom protein may suppress DNA amplification by interacting with nascent mutant primers and altering their conformation to permit more efficient kissing.

Materials and methods

Plasmids and bacteria

The bacterial host for these experiments was the *E.coli* K12 derivative DG75, which has been previously described (Fitzwater *et al.*, 1988). The parent plasmid is pNOP42, a 4.2 kb plasmid containing a wild-type ColE1 replication origin and carrying the *bla* gene (Muesing *et al.*, 1981). Isolation and characterization of pMM1, pMM4, pMM7 and pEW2705 has been described previously (Wong *et al.*, 1982). The plasmid used to provide the *rop* gene product in *trans* was pGC8, which has a plasmid R6K origin and a 2.2 kb *Eco*RI fragment from pBR322 carrying the *rop* gene region (Cesareni *et al.*, 1984). A derivative of pGC8 lacking the *rop* gene was constructed by removing the *Eco*RI fragment; this plasmid was designated pTF487.

Cells were grown in 2XYT medium containing 100 μg/ml ampicillin as described (Fitzwater *et al.*, 1988). Cleared lysates for copy number determinations were prepared by the alkaline method as previously described (Fitzwater *et al.*, 1988).

Site-directed mutagenesis

Mutations were site directed using the method of Kunkel *et al.* (1987). Oligonucleotides carrying the desired change were annealed to single-stranded DNA from a derivative called pSTK131, a 3.6 kb plasmid derived from pNOP42 which contains the wild-type ColE1 replication origin and the phage f1 replication origin. After annealing, primer extension and ligation, the DNA was used to transform the *dut ung* strain CJ236. Transformants carrying plasmids with the desired changes were identified by the presence of novel restriction sites created by the change; 128G creates an *Fnu*4H1 site, 131A creates an *Acc*I site.

Preparation of RNA I

Uniformly labeled RNA I was prepared by *in vitro* transcription of supercoiled plasmid DNA. A 500 μl reaction mixture contained 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM potassium glutamate, 1 mM dithiothreiotol, 200 μM each of ATP, CTP, GTP and UTP, 26 units/ml of *E.coli* RNA polymerase (Pharmacia), 0.87 μM [α-^{32}P]ATP (410 Ci/mmol, Amersham), and 60 μg/ml of pEW2705 DNA. Reactions were carried out for 90 min at 37°C and terminated by phenol extraction. After ethanol precipitation, the RNA pellet was dissolved in water and loading buffer (83% formamide, 17 mM EDTA, 0.05% bromophenol blue–xylene cyanol), boiled for 3 min, then loaded on a 6% polyacrylamide gel containing 7 M urea. Electrophoresis was carried out in 1 × Tris-borate buffer at 35 W for 6 h. RNA I was eluted by diffusion, ethanol precipitation and resuspended in water.

Primer transcription

Primer RNA was selectively transcribed and labeled at the 5′-terminal region by the procedure of Masukata and Tomizawa (1984) with modifications described in Fitzwater *et al.* (1988). The DNA templates for transcription were supercoiled DNAs that were phenol extracted after twice banding in CsCl gradients.

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References

Cesareni,G.C., Cornelissen,M., Lacatena,R.M. and Castagnoli,L. (1984) *EMBO J.*, **3**, 1365–1369.

Dooley,T.P. and Polisky,B. (1987) *Plasmid*, **18**, 24–34.

Fitzwater,T., Zhang,X.Y. and Polisky,B. (1988) *EMBO J.*, **7**, 3289–3297.

Freier,S.M., Kierzek,R., Jaeger,J., Sugimoto,N., Caruthers,M.H., Nelson,T. and Turner,D.H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9373–9377.

Itoh,T. and Tomizawa,J. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 2450–2454.

Kassavetis,G.A. and Chamberlin,M.J. (1981) *J. Biol. Chem.*, **256**, 2777–2786.
Kramer,F.R. and Mills,D.R. (1981) *Nucleic Acids Res.*, **9**, 5109–5124.
Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367–382.
Lacatena,R.M. and Cesareni,G. (1981) *Nature*, **294**, 623–626.
Landick,R. and Yanofsky,C. (1987) In Neidhardt,F.C. (eds), *Escherichia coli and Salmonella typhimurium*. ASM Washington, DC, pp. 1276–1301.
Levin,J.R. and Chamberlin,M.J. (1987) *J. Mol. Biol.*, **196**, 61–84.
Masukata,H. and Tomizawa,J. (1984) *Cell*, **36**, 513–522.
Masukata,H. and Tomizawa,J. (1986) *Cell*, **44**, 125–136.
Morita,M. and Oka,A. (1979) *Eur. J. Biochem.*, **97**, 435–443.
Polisky,B. (1988) *Cell*, **55**, 929–932.
Selzer,G., Som,T., Itoh,T. and Tomizawa,J. (1983) *Cell*, **32**, 119–129.
Simons,R.W. and Kleckner,W. (1988) *Annu. Rev. Genet.*, **22**, 567–600.
Tamm,J. and Polisky,B. (1983) *Nucleic Acids Res.*, **11**, 6381–6397.
Tamm,J. and Polisky,B. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 2257–2261.
Tomizawa,J. (1984) *Cell*, **38**, 861–870.
Tomizawa,J. (1985) *Cell*, **40**, 527–535.
Tomizawa,J. (1986) *Cell*, **47**, 89–97.
Tomizawa,J. and Itoh,T. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6096–6100.
Tomizawa,J. and Som,T. (1984) *Cell*, **38**, 871–878.
Turner,D.H., Sugimoto,N. and Freier,S.M. (1988) *Annu. Rev. Biophys. Biophys. Chem.*, **17**, 167–192.
Wong,E.M. and Polisky,B. (1985) *Cell*, **42**, 959–966.
Wong,E.M., Muesing,M.A. and Polisky,B. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 3570–3574.
Zuker,M. and Steigler,M. (1981) *Nucleic Acids Res.*, **9**, 133–148.

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