Research Article

Specific Interactions in RNA Enzyme-Substrate Complexes

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Analysis of crosslinked complexes of M1 RNA, the catalytic RNA subunit of ribonuclease P from *Escherichia coli*, and transfer RNA precursor substrates has led to the identification of regions in the enzyme and in the substrate that are in close physical proximity to each other. The nucleotide in M1 RNA, residue C92, which participates in a crosslink with the substrate was deleted and the resulting mutant M1 RNA was shown to cleave substrates lacking the 3′ terminal CCAUCA sequence at sites several nucleotides away from the normal site of cleavage. The presence or absence of the 3′ terminal CCAUCA sequence in transfer RNA precursor substrates markedly affects the way in which these substrates interact with the catalytic RNA in the enzyme-substrate complex. The contacts between wild-type M1 RNA and its substrate are in a region that resembles part of the transfer RNA "E" (exit) site in 23*S* ribosomal RNA. These data demonstrate that in RNA's with very different cellular functions, there are domains with similar structural and functional properties and that there is a nucleotide in M1 RNA that affects the site of cleavage by the enzyme.

SEVERAL RNA'S HAVE ENDONUCLEOLYTIC OR LIGATION ACtivities (*1*). The mechanisms of cleavages by representatives of the three main classes of these RNA's, namely (i) group I introns, (ii) plant viroid or virusoid RNA, and (iii) M1 RNA, the catalytic subunit of *Escherichia coli* ribonuclease P, are different from one another. In the first two classes, the catalytic site can be localized to a stretch of contiguous nucleotides or a particular feature of secondary structure; as to M1 RNA, little is known about its active site either when it acts alone (*2*) or when it acts in the holoenzyme complex with its protein cofactor (*2, 3*). The intact enzyme can cleave transfer RNA precursor substrates and the cleavage appears to depend on interactions between the substrate and several regions of the M1 RNA molecule that must be brought together by three-dimensional folding (*4, 5*). To elucidate in greater detail some aspects of structure-function relationships in M1 RNA, we have studied a site in M1 RNA that can be crosslinked by ultraviolet irradiation to a tRNA precursor substrate. In contrast to protein enzymes, RNA enzymes lend themselves, through the use of reverse transcriptase, to studies of the details of enzyme-substrate contacts.

Analysis of a crosslinked enzyme-substrate complex. To identify the region in M1 RNA that is in close proximity to its substrate, a series of ultraviolet (UV) light–mediated crosslinking experiments was performed initially with M1 RNA and a substrate [pTyr:Ssp, the precursor to $tRNA^{Tyr}$ with 43 extra nucleotides at its 3′ end (Fig. 1) (*6, 7*)] that has a lower K_m (five times) and a lower k_{cat} (100 times) than the wild-type substrate (pTyr, the precursor to $tRNA^{Tyr}$ from *Escherichia coli* found in vivo). This substrate, which forms stable complexes with M1 RNA (*7*), gives a 33 percent higher yield of enzyme-substrate complex when mixed with M1 RNA than does the wild-type substrate (pTyr) in Cs_2SO_4 gradients (*8*).

Mixtures of M1 RNA and pTyr:Ssp were irradiated with UV light under conditions that generate intramolecular crosslinks in various RNA molecules including M1 RNA (*9*). In experiments with UV light at 300 or 254 nm, crosslinks between enzyme and substrate were formed (Fig. 2). The RNA's migrating in the positions of bands a, b, c (lanes 1 and 3), and d (lanes 2 and 4) were recovered from the gel and analyzed on denaturing and nondenaturing polyacrylamide gels (species b, c, and d are absent in the nonirradiated control samples). On the basis of the composition of the reaction mixtures and analysis of the electrophoretic mobility of the isolated species in gels (*10*), we have determined that bands a and a′ correspond to non-crosslinked RNA, bands b and b′ correspond to intramolecular crosslinked species, bands c and c′ to intermolecular crosslinked RNA (that is, the same species of RNA as that found in dimers or multimers) and bands d and d′ correspond to crosslinked enzyme-substrate complex.

Crosslinked, unlabeled enzyme-substrate complex was purified from gel slices and analyzed, with reverse transcriptase, by extension from deoxyribonucleotide primers complementary to M1 RNA and to pTyr:Ssp (*11, 12*). Identical experiments were also performed with other derivatives of the precursor to $tRNA^{Tyr}$, lacking the 3′ terminal UCA (pTyr − UCA) or CCAUCA (pTyr − CCAUCA) sequence (Fig. 1), but the enzyme-substrate complex was prepared under slightly different conditions in these cases (*9*). Reverse transcriptase chain terminates all along the sequence of both M1 RNA, and the substrates and these events can be seen as faint bands in the lanes that contain exclusively M1 RNA that has been irradiated but that has the same mobility as unirradiated M1 RNA (Fig. 3A, lanes 2 and 3; Fig. 3C, lanes 3 and 4) and in the lane that contains just the substrate (Fig. 3B, lane 2; Fig. 3D, lanes 3 and 4). Similar behavior of reverse transcriptase with other RNA molecules has been observed (*12*). Some of the chain terminations seen in Fig. 3 may reflect gross changes in the conformation of the RNA that

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occur in the enzyme-substrate complex and are frozen by the crosslinking. However, we assume that chain termination events characteristic of the enzyme-substrate complexes are consequences of nucleotide modification by crosslinks at the position adjacent to the chain termination site (*13*).

When primer extension analysis was performed on the enzyme-substrate complex (with pTyr:Ssp or pTyr − UCA as substrates, Fig. 1), with a primer complementary to nucleotides 220 to 236 of M1 RNA (Fig. 3A, lane 1; Fig. 3C, lane 1), a chain termination event during reverse transcription, not seen in the case of extensions of M1 RNA alone, was observed (*14*). This chain termination occurs at C93 in M1 RNA, indicating that nucleotide C92 is crosslinked to the substrate. We then deleted nucleotide C92 from M1 RNA (*15*) to determine its effect on the function of M1 RNA and on the location of the crosslink with the substrate (because of the adjacent C's in the sequence C92 C93, the exact identity of the deleted nucleotide cannot be determined). A double deletion mutation, at positions C92 and G106, was also isolated during the original search for Δ92 (there are seven consecutive G's from positions 105 to 111 in M1 RNA: the deleted G has been assigned to the position that is base-paired to C92). Primer extension analysis was therefore carried out on crosslinked complexes made with Δ92 M1 RNA or Δ92,106 M1 RNA and pTyr:Ssp as substrate. The results were the same with both mutant derivatives of M1 RNA and are summarized along with other data in Table 1. The data shown in Fig. 3, A and B, were obtained with Δ92,106 M1 RNA. The appearance of a new chain termination at A93 is visible in the complex with mutated RNA (Fig. 3A, lane 4). In the single Δ92 mutant the chain termination occurred at A93 as it did with pTyr − UCA as substrate (Fig. 3C, lane 2), indicating that the extra 3′ flanking sequence did not affect the position of the crosslink (*8*).

To determine the location in the substrate of the other end of the crosslink, we used oligonucleotides complementary to the 3′ end of the substrate as primers for reverse transcription. An efficient chain termination was observed with M1 RNA and Δ92,106 M1 RNA at position −2 (G −2; Fig. 3B, lanes 1 and 3). The same chain termination at position −2 was also observed with Δ92 M1 RNA (*8*). Therefore, the nucleotide in the substrate that is crosslinked with the enzyme is C −3. In every experiment illustrated in Fig. 3, the putative crosslink formed between the enzyme and the substrate makes a pyrimidine photodimer.

Enzyme-substrate complexes made with other variants of M1 RNA, which contain mutations located near C92 and elsewhere (Fig. 1), were analyzed in a similar fashion (*16*). Mutants U93 M1

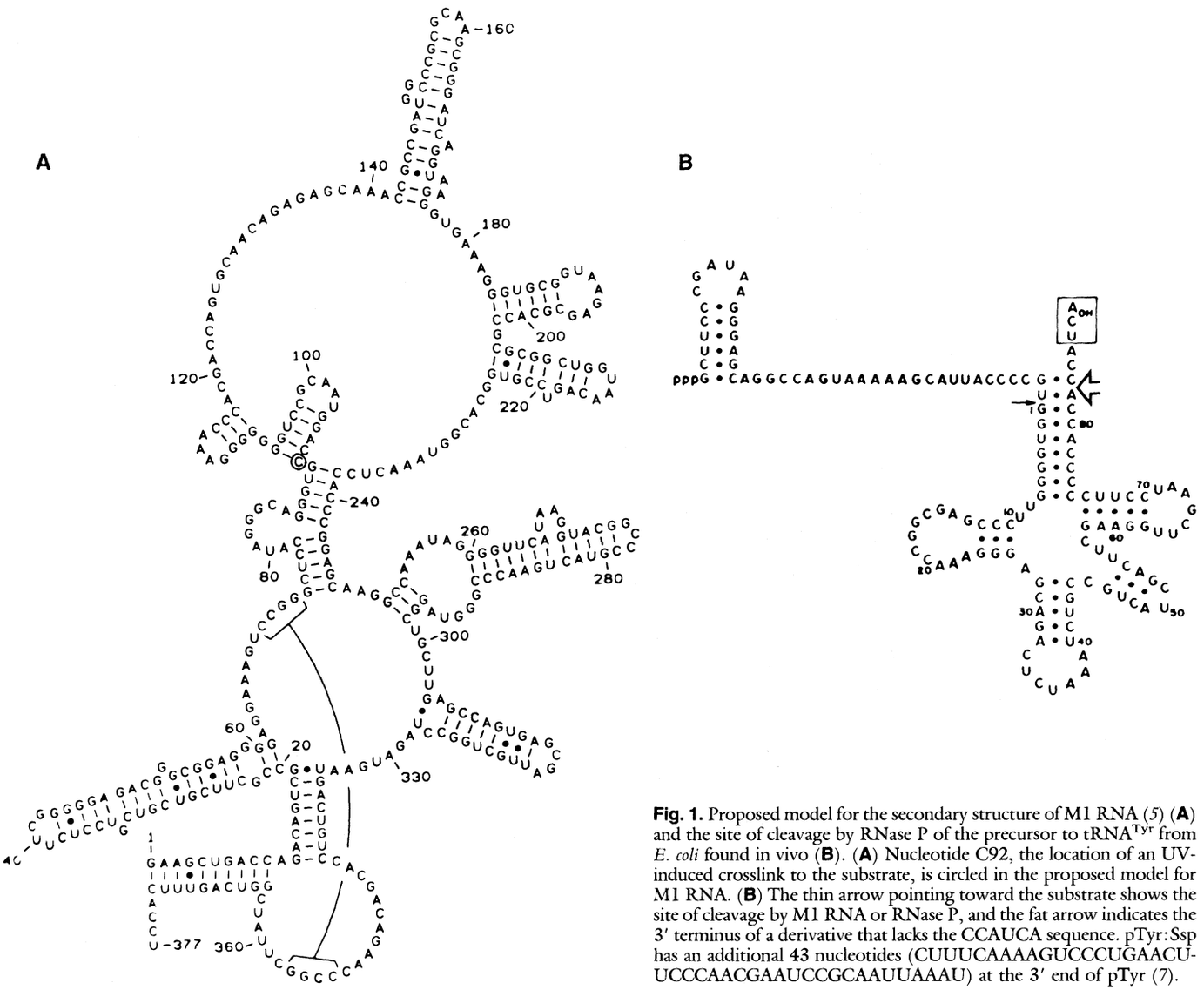

Fig. 1. Proposed model for the secondary structure of M1 RNA (*5*) (**A**) and the site of cleavage by RNase P of the precursor to $tRNA^{Tyr}$ from *E. coli* found in vivo (**B**). (**A**) Nucleotide C92, the location of an UV-induced crosslink to the substrate, is circled in the proposed model for M1 RNA. (**B**) The thin arrow pointing toward the substrate shows the site of cleavage by M1 RNA or RNase P, and the fat arrow indicates the 3′ terminus of a derivative that lacks the CCAUCA sequence. pTyr:Ssp has an additional 43 nucleotides (CUUUCAAAAGUCCCUGAACU-UCCCAACGAAUCCGCAAUUAAAU) at the 3′ end of pTyr (*7*).

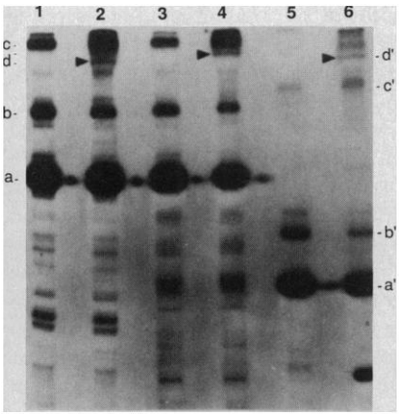

Fig. 2. Identification of RNA crosslinked by UV irradiation. End-labeled RNA's were irradiated as described in (*9*) and analyzed on a 5 percent polyacrylamide sequencing gel that contained 7*M* urea. (Lane 1) M1 RNA, 5′ end-labeled with [γ-^{32}P] ATP (a total of 430 ng; 1.9 × 10^6 cpm) alone; (lane 2) same as in lane 1, but incubated in the presence of unlabeled pTyr:Ssp (200 ng); (lane 3) M1 RNA, 3′ end-labeled with ^{32}P-pCp (a total of 300 ng; 1.2 × 10^6 cpm) alone; (lane 4) same as in lane 3 but incubated in the presence of unlabeled pTyr:Ssp (200 ng); (lane 5) pTyr:Ssp, 3′ end-labeled with ^{32}P-pCp (a total of 230 ng; 1 × 10^6 cpm) alone; (lane 6) same as in lane 5 but incubated in the presence of unlabeled M1 RNA (235 ng). (Bands a, a′) Non-crosslinked species of RNA; (b, b′) intramolecularly crosslinked RNA's; (c, c′) intermolecularly crosslinked RNA's; and (d, d′) crosslinked E–S complex. The arrows indicate the positions of band d in lanes 2 and 4 and band d′ in lane 6. Breakdown products of end-labeled RNA's are evident. The dark band at the bottom of lane 6 corresponds to the cleavage product, containing the tRNA sequence, of the transcript of pTyr:Ssp.

RNA and A106 M1 RNA (*4, 14*) also generated the chain termination at position 93 and an additional increase was observed in the intensity of a band that corresponded to a chain termination at G68, whereas mutants G65 M1 RNA and Δ65 M1 RNA gave a pattern that resembled the one obtained with wild-type M1 RNA. Furthermore, U93 M1 RNA and A106 M1 RNA made a stronger contact with U − 8 in the substrate than did wild-type M1 RNA, G65 M1 RNA, or Δ65 M1 RNA. A deletion mutation of M1 RNA, at position G91 (Δ91 M1 RNA), gave a low yield of enzyme-substrate complex and a corresponding decrease in intensity of the

Table 1. Enzyme-substrate contacts.

Complex	Contacts in		Cleavage site
	Enzyme	Substrate	
M1–pTyr:Ssp	C92	C − 3	G1
Δ92–pTyr:Ssp	C92	C − 3	G1
M1–pTyr − CCAUCA	U69 > U40	U − 1 > U − 8	G1
Δ92–pTyr − CCAUCA	U69 > U40 >U126	U − 1 > U − 8	C − 4, C − 5, C − 6
M1–pTyr − CCAUCA*	U69 < U40	U − 1 ~ U − 8	G1
Δ92–pTyr − CCAUCA*	U69 ~ U40	U − 1 > U − 8	G1

*In buffers containing 3*M* ammonium acetate and 50 m*M* $MgCl_2$.

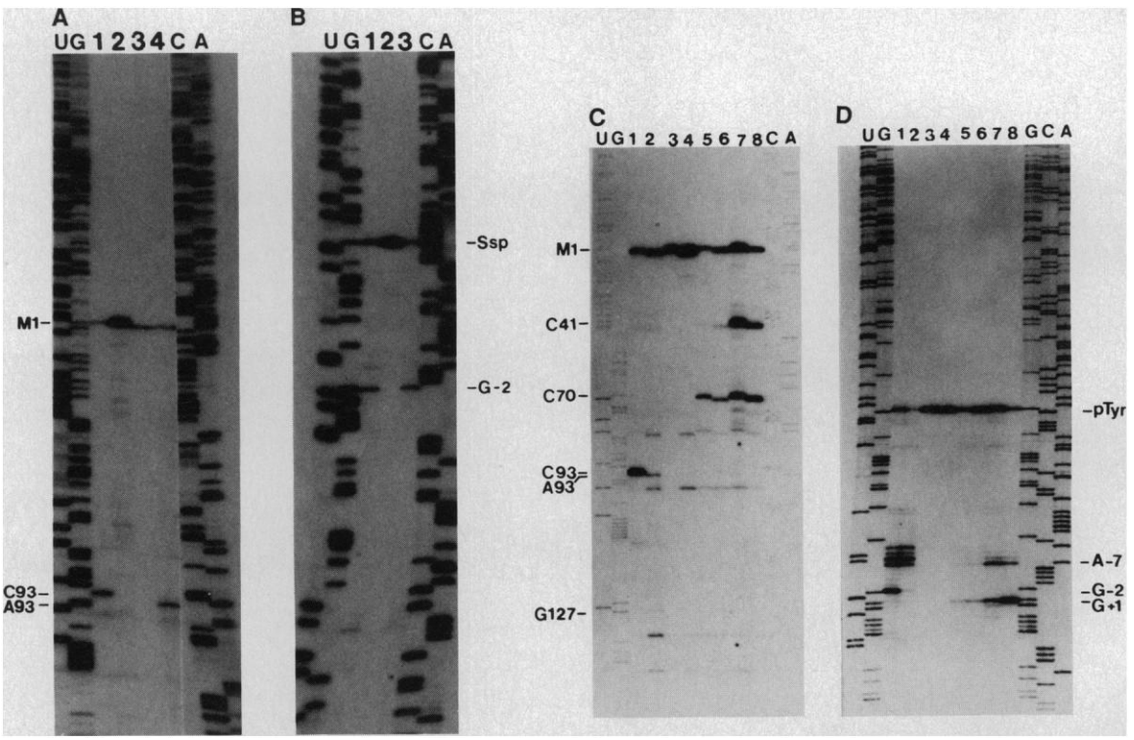

Fig. 3. Reverse transcription analysis of enzyme-substrate complexes. Analyses were performed as described in the text and in (*12, 13*). Lanes labeled A, C, G, U are reference analyses of DNA sequences (*14*) corresponding to the appropriate RNA templates. The ddTTP tracks are labeled U to facilitate comparisons with RNA sequences. Primers complementary to nucleotides 220 to 236 in M1 RNA and Δ92 M1 RNA (**A** and **C**), nucleotides 104 to 123 in pTyr:Ssp (**B**), and nucleotides 75 to 82 in pTyr − CCAUCA (**D**) were used. Only critical segments of the autoradiographs are shown. (**A**) Priming from the enzyme. (Lane 1) Analysis of the complex between M1 RNA and pTyr:Ssp; (lane 2) M1 RNA alone; (lane 3) Δ92,106 M1 RNA alone; (lane 4) complex of Δ92,106 M1 RNA and pTyr:Ssp. (**B**) Priming from the substrate. (Lane 1) Complex of pTyr:Ssp and M1 RNA; (lane 2) pTyr:Ssp alone; (lane 3) complex of pTyr:Ssp and Δ92,106 M1 RNA. (**C**) Priming from the enzyme. (Lane 1) Complex of M1 RNA and pTyr − UCA; (lane 2) complex of Δ92 M1 RNA and pTyr − UCA; (lane 3) M1 RNA alone; (lane 4) Δ92 M1 RNA alone; (lane 5) complex of M1 RNA and pTyr − CCAUCA; (lane 6) complex of Δ92 M1 RNA and pTyr − CCAUCA; (lane 7) complex of M1 RNA and pTyr − CCAUCA (in 3*M* buffer consisting of 50 m*M* tris-HCl, *p*H 7.5, 50 m*M* $MgCl_2$, 3*M* ammonium acetate); (lane 8) complex of Δ92 M1 RNA and pTyr − CCAUCA (in 3*M* buffer). (**D**) Priming from the substrate. (Lane 1) Complex of pTyr − UCA and M1 RNA; (lane 2) complex of pTyr+CCA and Δ92 M1 RNA; (lane 3) pTyr − UCA alone; (lane 4) pTyr − CCAUCA alone; (lane 5) complex of M1 RNA and pTyr − CCAUCA; (lane 6) complex of pTyr − CCAUCA and Δ92 M1 RNA; (lane 7) complex of pTyr − CCAUCA and M1 RNA (in 3*M* buffer); (lane 8) complex of pTyr − CCAUCA and Δ92 M1 RNA (in 3*M* buffer). The positions of certain nucleotides and full-length transcripts of the enzyme (M1) or substrates (Ssp and pTyr) are marked. Both Δ92 M1 RNA, which is one nucleotide shorter than M1 RNA, and Δ92,106 M1 RNA, which is two nucleotides shorter than M1 RNA, have a slightly greater mobility than M1 RNA. A93 (A and C) corresponds to the pause generated by reverse transcriptase in the complexes Δ92,106 M1 RNA/pTyr:Ssp and Δ92 M1 RNA/pTyr − UCA, respectively.

bands produced by chain termination events.

Phenotypes of mutants in vitro. If nucleotide C92 is of critical importance in enzyme-substrate interactions, deletion of this nucleotide should lead to a variant of M1 RNA with an altered phenotype in vitro. When M1 RNA and its derivatives were assayed alone for activity in vitro with our standard substrate, pTyr (Fig. 1), Δ92 M1 RNA exhibited a severely defective phenotype (Table 2). The K_m was 100 times higher and the k_{cat} was six times lower than wild type. When this and other deletion and base substitution mutants were assayed in the holoenzyme complex with C5 protein, the deletion mutants were generally more severely affected, but all the mutants had k_{cat}/K_m ratios that were from 1 to 10 percent that of wild type (Table 2). The behavior of Δ92 M1 RNA is not particularly distinctive in these assays. All the deletion mutants, in contrast to the base substitution mutants, have drastically diminished activity when the mutated RNA is tested alone, indicating that the maintenance of certain spatial relationships in the structure of M1 RNA is more important than the actual identity of the nucleotides at positions 65, 91, and 92. More direct support for the hypothesis that C92, in particular, plays a critical role in the action of M1 RNA is derived from studies of the reaction of Δ92 M1 RNA and Δ92,106 M1 RNA with another substrate of particular interest.

The presence or absence of the CCA 3′ terminal sequence in tRNA precursors can markedly affect their susceptibility to cleavage by M1 RNA (*17*), presumably because this common sequence is found adjacent to the cleavage site (Fig. 1) and is important either for binding of substrate or departure of product from the complex with the enzyme. Therefore, we assayed the mutant M1 RNA's with a substrate that lacked the 3′ terminal CCA sequence (called pTyr − CCAUCA to indicate that it is missing the last six nucleotides present in the wild-type substrate, pTyr, found in vivo, in *E. coli*). To our surprise, we found that Δ92 M1 RNA and Δ92,106 M1 RNA only among the mutants listed in Table 2, now cleaved pTyr − CCAUCA (*18*) (Fig. 1) at sites four, five, and six nucleotides upstream from the normal site of cleavage (Fig. 4). This novel result is indicative of the ability of a derivative of M1 RNA to cleave a substrate at a site other than the site cleaved by wild-type M1 RNA. Another substrate lacking the 3′ terminal CCA sequence, a precursor to $tRNA^{Ser}$ from *Saccharomyces cerevisiae* (*19*), was also assayed with Δ92 M1 RNA and Δ92,106 M1 RNA, and aberrant cleavage at a site four nucleotides upstream from the normal site of cleavage was detected (*8*).

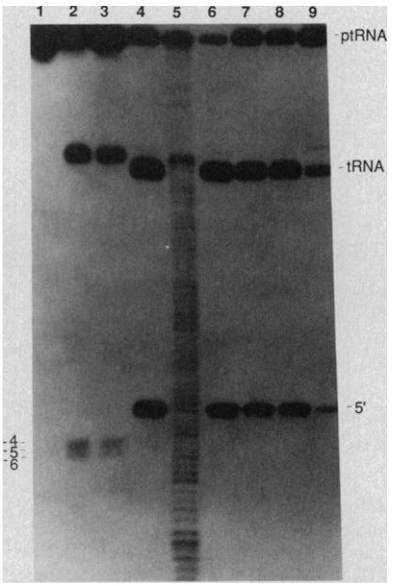

Fig. 4. Cleavage of pTyr − CCAUCA by Δ92 M1 RNA. RNase P activity was assayed after a 15-minute incubation at 37°C. The substrate, ^{32}P-labeled pTyr − CCAUCA ($2 \times 10^{-6}M$) was incubated with wild-type M1 RNA ($4 \times 10^{-8}M$) or Δ92 M1 RNA ($1.7 \times 10^{-6}M$) alone, (lanes 2, 3, 7 to 9), or with C5 protein in addition to the catalytic RNA (lanes 4 and 6) as indicated. After ethanol precipitation, the reaction products were resolved on an 8 percent polyacrylamide sequencing gel. The source of enzyme and buffer conditions in each reaction were as follows. (Lane 1) No enzyme added: buffer A [50 m*M* tris-HCl, *p*H 7.5, 100 m*M* $MgCl_2$, 100 m*M* NH_4Cl, 4% (w/v) poly(ethyleneglycol)]; (lane 2) Δ92 M1 RNA, buffer B (50 m*M* tris-HCl, *p*H 7.5, 10 m*M* $MnCl_2$, 10 m*M* spermidine, 100 m*M* NH_4Cl); (lane 3) Δ92 M1 RNA, buffer A; (lane 4) Δ92 M1 RNA ($1 \times 10^{-8}M$) and C5 protein ($1 \times 10^{-7}M$), buffer C (50 m*M* tris-HCl, *p*H 7.5, 10 m*M* $MgCl_2$, 100 m*M* NH_4Cl); (lane 5) partial alkaline digest of 3′ end-labeled pTyr − CCAUCA; (lane 6) M1 RNA ($1 \times 10^{-8}M$) and C5 protein ($1 \times 10^{-7}M$): buffer C; (lane 7) M1 RNA, buffer A; (lane 8) M1 RNA, buffer B; (lane 9) M1 RNA, buffer D (50 m*M* tris-HCl, *p*H 7.5, 10 m*M* $MgCl_2$, 10 m*M* spermidine, 100 m*M* NH_4Cl).

When the holoenzyme made with Δ92 M1 RNA or Δ92,106 M1 RNA was used in these assays, or when the reactions were performed in buffers that contained 3*M* ammonium acetate, cleavage occurred predominantly at the normal site at nucleotide 1 of the mature tRNA sequence of pTyr − CCAUCA, although some residual cleavage still occurred at the unusual sites (*20*). Spermidine cannot replace C5 protein, the protein subunit of ribonuclease (RNase) P, in correcting the aberrant cleavage by Δ92 M1 RNA (Fig. 4, lanes 2 and 4). The normal reaction, governed by wild-type M1 RNA, proceeds more rapidly in the presence of $MnCl_2$ and spermidine than it does in the presence of $MgCl_2$ and spermidine (Fig. 4, lanes 8 and 9).

Contacts between M1 RNA or Δ92 M1 RNA and pTyr − CCAUCA. The unexpected properties of the cleavage of pTyr − CCAUCA by Δ92 M1 RNA might be the result of contacts between the mutant M1 RNA and the substrate that differ from those made by wild-type M1 RNA. Indeed, two strong chain termination events in the reverse transcriptional analysis of Δ92 M1 RNA in the complex with pTyr − CCAUCA were found at nucleotides C70 and C41, and a weaker one was seen at G127 (Fig. 3C, lane 6). The interaction of pTyr − CCAUCA with Δ92 M1 RNA, therefore, is different from that of pTyr:Ssp. Surprisingly, the latter statement is also true for the contacts formed by pTyr − CCAUCA with wild-type M1 RNA (Fig. 3C, lane 5). In this case, however, the strongest chain termination is visible at C70 and a weaker one is seen at C41. A minor chain termination is also apparent at G127. When the same experiment is repeated with wild-type or Δ92 M1 RNA in buffers that contained 3*M* ammonium acetate to mimic conditions under

Table 2. Kinetic parameters of the reactions of M1 RNA and several variants with pTyr. The parameters were determined for reactions carried out as described (*2, 4*). pTyr was produced by transcription in vitro by T7 RNA polymerase. For reactions with RNase P, the holoenzyme was formed, by reconstitution, from the subunits (*2*) at a molar ratio of C5 protein to M1 RNA of 20:1. In reactions with M1 RNA alone, the concentration of M1 RNA was $2 \times 10^{-8}M$. In reactions with M1 RNA plus C5 protein, the concentration of M1 RNA was $5 \times 10^{-10}M$. The amount of mutant M1 RNA's used in reactions with holoenzyme varied from $2.5 \times 10^{-9}M$ to $2 \times 10^{-8}M$. The substrate (pTyr) was always present in molar excess.

Enzyme		K_m	k_{cat}*	k_{cat}/K_m
RNA	Protein	($10^{-7}M$)		($10^{-7}M$)
M1	None	0.3	0.4	1.3
Δ92	None	40	0.07	0.002
Δ92,106	None	67	0.12	0.002
Δ91	None	3.3	0.3	0.1
Δ65†	None		0	
M1	C5	0.33	29	87
Δ92	C5	0.4	2.7	6.7
Δ92,106	C5	1	1.3	1.3
U93	C5	5	40	8
Δ91	C5	5	3.6	0.7
A106	C5	5	53	10.6
Δ65	C5	2	2.5	1.3
G65	C5	5	25	5
C65	C5	3.3	32	9.7

*k_{cat} is given in moles of product per mole of enzyme per minute. †Δ65 M1 RNA showed no detectable activity (<0.01 percent) when compared to wild-type M1 RNA.

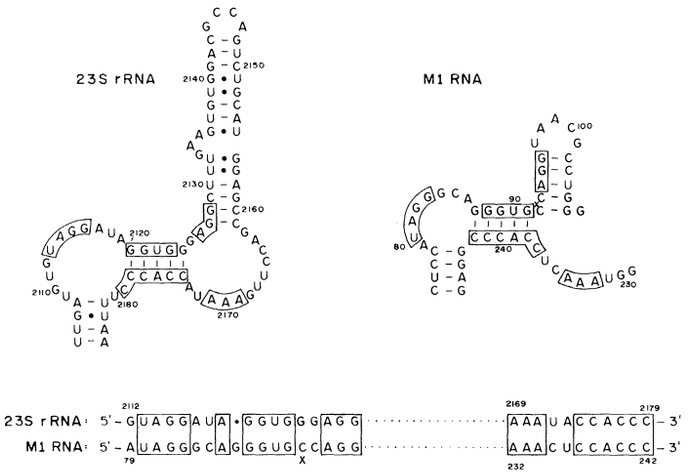

Fig. 5. Comparison of part of the E site of 23*S* rRNA with a region in M1 RNA that surrounds the crosslink with the substrate. The secondary structures are taken from Moazed and Noller (*21*) and James *et al.* (*5*). The x marks C92, the nucleotide in M1 RNA that is crosslinked to the substrate. Nucleotides shown in boxes are found in approximately the same relative positions in the structures shown.

which Δ92 M1 RNA and Δ92,106 M1 RNA cleave substrates at the normal site, the chain terminations at C70 and C41 are present but the one at G127 is totally absent from the complex made with Δ92 M1 RNA (Fig. 3C, lane 8). While M1 RNA and Δ92 M1 RNA exhibit somewhat different intensities in the patterns of chain termination events (Fig. 3), it appears that the nature of the substrate itself has also induced a major change in the contacts on the enzyme—from those in the region near C92 to those in other parts of the enzyme as portrayed in the two-dimensional model shown in Fig. 1. We therefore anticipated that the crosslinks of the RNA enzymes made with pTyr − CCAUCA, at the very least in the case of Δ92 M1 RNA, would be different from those seen with pTyr:Ssp.

Reverse transcription of pTyr − CCAUCA in the enzyme-substrate complex shows that Δ92 M1 RNA makes crosslinks with pTyr − CCAUCA at more than a single site. The most efficient chain termination now occurs at G1 in the substrate and another one is seen at −7, as before. Other weaker chain termination events in this region of the substrate are also apparent (Fig. 3D), as if the enzyme-substrate complex can exist in several states, only one of which leads to the productive cleavage event at positions −4, −5, and −6. The complex made with wild-type M1 RNA showed the same pattern. In buffers containing 3*M* ammonium acetate, the chain termination event at G1 became by far the predominant one (the crosslinked nucleotide in this case would be U −1). These observations are different from the results obtained with pTyr:Ssp, in which case when cleavage was observed at the normal site, nucleotide G1, a single strong chain termination was evident in the substrate near the site of cleavage (position −2). The primers used for these reverse transcription experiments with pTyr − CCAUCA were complementary to nucleotides 75 to 82 in the tRNA acceptor stem (unlike the primer used for pTyr:Ssp, which hybridized only to the extra 3′ flanking sequence). If a critical contact was made with the substrate in the 3′ part of the acceptor or T stem, we would not have detected it.

A domain in M1 RNA resembling the ribosomal E site. We have studied the only covalently joined enzyme-substrate complexes found after UV irradiation of mixtures of M1 RNA or other derivatives of it and wild-type substrate. Our results show that the region around nucleotide C92 in wild-type M1 RNA is important for interactions specifically and particularly with the nucleotides near the site of cleavage of tRNA precursor substrates. In addition, the remarkable resemblance of this region to part of the tRNA binding "E" (exit) site on 23*S* RNA (*21*) from *E. coli* (Fig. 5) gives further weight to the more specific proposal that the region around nucleotide C92 in M1 RNA is a binding site for the acceptor stem of the tRNA moiety of tRNA precursors. The region of 23*S* ribosomal RNA (rRNA) shown in Fig. 5 encompasses most of the nucleotides protected from chemical modification by the binding of deacylated tRNA to ribosomes prior to the release of the tRNA from the translation machinery (*21*). The sequence between positions 232 and 242 in M1 RNA is almost identical to nucleotides 2169 to 2179

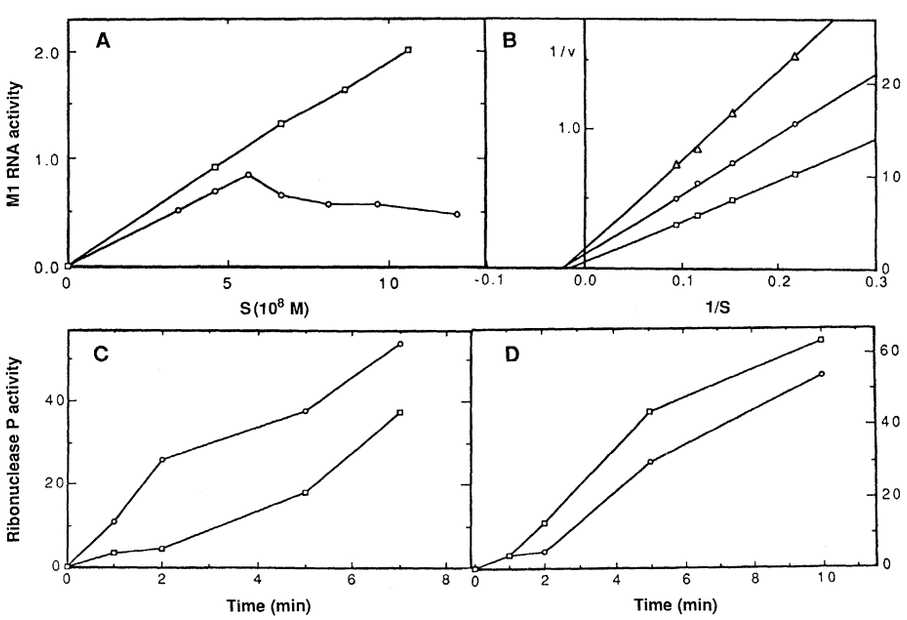

Fig. 6. Inhibition of M1 RNA or RNase P activity. (**A**) Effect of substrate concentration on M1 RNA activity. M1 RNA ($2 \times 10^{-8}M$) was first incubated for 5 minutes at 37°C in buffer A (50 m*M* tris-HCl, *p*H 7.5, 100 m*M* NH_4Cl, 100 m*M* $MgCl_2$). Substrate [pTyr (○) or pTyr − CCAUCA (□)] was then added and samples were taken every minute for 6 minutes. Activity, expressed in picomoles of product per minute, was measured in the linear range of kinetics by separation of the components of the reaction mixture in an 8 percent polyacrylamide gel that contained 7*M* urea and densitometric analysis of the corresponding autoradiograms. (**B**) Lineweaver-Burk plot of the kinetics of M1 RNA activity with pTyr − CCAUCA as substrate in the presence or absence (circle; left-hand ordinate) of inhibitor. Reactions were carried out in buffer A with M1 RNA ($2 \times 10^{-8}M$) as enzyme, ^{32}P-labeled pTyr − CCAUCA as substrate, and unlabeled pTyr as inhibitor [$1 \times 10^{-7}M$ (□), $2 \times 10^{-7}M$ (△); right-hand ordinate). Units: $1/S = 10^8M^{-1}$; $1/v = 10^8M^{-1}$ min. (**C** and **D**) Effect of the tri(ribo) nucleotide CCA on M1 RNA activity. M1 RNA ($2 \times 10^{-8}M$) was first incubated in the presence (□) or absence (○) of tri(ribo)nucleotide (2 m*M*) for 5 minutes at 37°C as described above. Substrate ($4 \times 10^{-8}M$) was added (C, pTyr; D, pTyr − CCAUCA) and samples were taken as indicated in the figure. Activity is expressed as the percent of total substrate cleaved per minute.

of 23*S* rRNA, and there is also a high degree of similarity between nucleotides 79 to 96 of M1 RNA and 2112 to 2128 of 23*S* rRNA. The similar nucleotides in the two structures also have nearly the same relative location with respect to the loop and stem features. Such a resemblance between the two structures, although not exact in every detail, is sufficiently striking to provoke questions about similarities in function of each of these regions in their respective RNA's and, in particular, the binding or release (or both) of the tRNA moiety of the substrate from M1 RNA during the RNase P reaction (*22*). A comparison of the pattern of protection from chemical modification of M1 RNA in the crosslinked complex with pTyr with that of 23*S* rRNA in the complex with tRNA (*21*) should also be of interest.

The region in M1 RNA shown in Fig. 5 was initially examined because of the location at C92 of the crosslink with a substrate. The C5 protein subunit makes a footprint on M1 RNA that covers nucleotides 82 to 96 (*3*) and, when mixed with M1 RNA, yields holoenzyme that, in contrast to M1 RNA alone, carries out cleavage without regard to the presence or absence of the 3′ terminal CCA sequence in substrates (*17*).

The importance of the 3′ CCA sequence. That the acceptor stem of the substrate, specifically, is involved in binding to M1 RNA as it is to the E site in rRNA is demonstrated by experiments in which puromycin, an antibiotic analog of a 3′ terminal aminoacylated adenosine, has been shown to be an inhibitor (at a concentration of 2 to 3 m*M*) of the reaction carried out by RNase P (*23*). Furthermore, pTyr inhibits the reaction with itself as substrate at concentrations near $8 \times 10^{-8} M$, but when pTyr − CCAUCA is used as substrate, no such effect is observed (Fig. 6A). The amount of product produced in the reaction with pTyr is not enough to account for the inhibition. pTyr inhibits the reaction with pTyr − CCAUCA as substrate in a noncompetitive fashion as shown by the data in Fig. 6B and by other experiments in which we have measured V_{max} in the presence and absence of inhibitor (*8*). Effective binding of tRNA to the E site of 23*S* rRNA also requires an intact 3′ terminal sequence.

The noncompetitive nature of the inhibition of the reaction with pTyr − CCAUCA by pTyr suggests that the CCA binding site on M1 RNA is separated from the site that recognizes the folded structure of the intact tRNA moiety. This suggestion is consistent with other observations that we have made with mutants of M1 RNA and tRNA precursors (*8*). The importance of specific contacts between different segments of the tRNA moiety and M1 RNA is further confirmed by results of experiments in which we used the tri(ribo)nucleotide CCA as an inhibitor. At high concentrations (2 m*M*), as with puromycin, CCA inhibits the reaction with substrates that have an intact CCA sequence (Fig. 6C). However, this trinucleotide stimulates the reaction with substrates that lack the 3′ terminal CCA sequence (Fig. 6D). One interpretation of these results is that the trinucleotide, together with the rest of the tRNA precursor, fills the necessary binding sites on the surface of M1 RNA to create the illusion of an intact substrate which the enzyme can cleave. Similar phenomena have been observed by Masiakowski and Deutscher (*24*) in their studies of nucleotidyl transferase, an enzyme that adds nucleotides to the 3′ terminus of tRNA's, and by Renaud *et al.* (*25*) in studies of the aminoacyl synthetase reaction.

Substrate recognition by RNase P. We have now determined that an alteration in M1 RNA at the normal site of contact with substrates can result in a shift by at least four nucleotides of the location of the site of cleavage by the enzyme. Such a shift may imply a destabilization of the correct structure of M1 RNA and the existence of a measuring mechanism (*26*) that moves the acceptor stem of the substrate some fraction of a turn of an RNA helix relative to a point of contact of the tRNA sequence on the enzyme. If that is the case, our results show that the 3′ terminal CCA sequence must contain some of the reference marks for the measuring mechanism, at least for RNase P from *E. coli*. Other model substrates, with changes in the sequence of the mature tRNA, have also been shown to sustain shifts in the site of cleavage from the site expected with wild-type RNase P from *Xenopus laevis* (*27*).

Another explanation for our results is that the altered site of cleavage found with Δ92 M1 RNA may be a result of an "induced fit" of pTyr − CCAUCA on the mutant enzyme, which differs from that on the wild-type enzyme. Clearly, this aberrant induced fit can be reversed by the presence of the C5 protein or 3*M* ammonium acetate, an indication of the flexibility of the structure of the enzyme-substrate complex. It is known that C5 protein renders the holoenzyme sensitive to the fine details of the structure of the substrate (*28*) and enhances the reaction rate about 20-fold or more with all natural substrates. The protein cofactor has no noticeable effect on the site of cleavage by wild-type RNase P since wild-type M1 RNA always cleaves its substrates at the normal site, with the possible exception of the precursor to $tRNA^{His}$ which, alone among tRNA's, has eight base pairs in its acceptor stem (*29*). However, no alteration in the site of cleavage of substrate by a variant of M1 RNA, or the surprising effect on it of the protein cofactor, has previously been observed. Thus, these data and studies of inhibition of RNase P activity by oligonucleotides complementary to M1 RNA (*16*) show that the C5 protein has the ability to stabilize the correct structure of M1 RNA, in particular around the region of nucleotide C92, as described above, in a quite specific fashion, thereby restoring proper cleavage by certain mutants of M1 RNA.

The processes by which the tRNA acceptor stem binds to both M1 RNA and 23*S* rRNA of *E. coli* have a similar dependence on and sensitivity to the nature of the 3′ terminus of a tRNA structure. The apparent similarity of these phenomena may be limited to *E. coli* and some other bacteria in which all tRNA genes include the 3′ terminal sequences encoded in them (*30*). In many bacteria, such as the Archaebacteria and relatives of *B. subtilis*, the 3′ terminal CCA sequence is not always encoded in the tRNA genes and thus the way in which the acceptor stem of tRNA precursor molecules interacts with the RNA subunit of RNase P, as well as the structure of that RNA subunit itself, may be quite different from those features of the rRNA E site in the same organism.

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6. M1 RNA and pTyr:Ssp were prepared by transcription in vitro of DNA from the plasmids listed below. (i) Plasmid pT7M1 was constructed as follows: Plasmid p65M1s (the source of point mutations in the 5′ terminal portion of the gene that encodes M1 RNA; 4) was digested with Alu I and Ava I; plasmid pJA2′ [the source of the 3′ terminal portion of the gene that encodes for M1 RNA (*3*) and its transcription termination sequence] was digested with Ava I and Pst I. The purified DNA fragments were ligated to an oligonucleotide that contained the promoter of T7 polymerase and cloned between the Kpn I and Pst I sites of pUC19. (ii) Plasmid pT7Ssp, which was used to prepare the substrate for the UV crosslinking studies, was constructed as follows: A Pst I–EcoR I fragment derived from the Tyr T locus carried by bacteriophage $\phi 80su_3^+$ was first cloned into pBR322 (P. Minehart and S. Altman, unpublished). A Hha I–Hind III fragment (~205 bp) was subcloned from the derivative of pBR322 into the Sma–Hind III sites of pGem2 (Promega) to make pGem2/HH; pGem2/HH was digested with Ava I and Hind III and a resulting fragment of about 150 bp was purified and ligated to the large vector fragment obtained from digestion with Ava I and Hind III of pUC19TyrT [pUC19TyrT contains the sequence of the naturally occurring precursor to $tRNA^{Tyr}$, 131 nucleotides in length, adjacent to a bacteriophage T7 promoter (M. Baer and C. Guerrier-Takada, unpublished)]. DNA from the plasmid pT7Ssp, made by ligation of the two fragments described above, was digested with Ssp I prior to transcription in vitro by T7 RNA polymerase. The

resulting transcript (pTyr:Ssp) starts with the first nucleotide of the naturally occurring sequence of the precursor tRNA and is 174 nucleotides long. Some characteristics of this precursor tRNA as a substrate for RNase P are described in (7).
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8. C. Guerrier-Takada and S. Altman, unpublished results.
9. A. D. Branch, B. J. Benenfeld, H. D. Robertson, *Nucleic Acids Res.* **13**, 4889 (1985); A. D. Branch *et al.*, *Science* **243**, 649 (1989); A. D. Branch, B. J. Benenfeld, C. P. Paul, H. D. Robertson, *Methods Enzymol.* **180**, 418 (1989). Complexes were studied as follows: M1 RNA (wild-type or mutant) (250 pmol) made by transcription in vitro were combined with an excess (2 to 4 molar) of pTyr:Ssp or pTyr − UCA in a buffer that contained 100 m*M* $MgCl_2$, 50 m*M* tris. HCl, *p*H 7.5, and 100 m*M* NH_4Cl, in a volume of 10 to 50 μl, as appropriate, and incubated at 37°C for 15 minutes to allow the formation of the enzyme-substrate complex. The mixture was placed on the surface of a UV (254 nm) transilluminator (UVP Inc. or Fotodyne) in a single drop, and irradiated for 3 minutes at room temperature. The irradiated samples were then subjected to electrophoresis in denaturing polyacrylamide gels and the species of interest were purified from gel slices. When complexes were prepared with substrate that lacked CCA (pTyr − CCAUCA), M1 RNA (wild type or mutant) was first incubated alone for 5 minutes at 37°C in the same buffer described above. Substrate was then added, and the reaction mixture was immediately irradiated as described. The efficiency of enzyme-substrate complex formation was at least three to four times greater with substrates without CCA. The use of this procedure for CCA containing substrates resulted in complete absence of enzyme-substrate complex formation, thus indicating that, when CCA is present in the substrate, some conformational change has to occur in the molecule before the substrate is available for interaction with the enzyme.
10. The identities of the RNA species in the bands shown in Fig. 2 were determined (i) by comparing mobilities of the isolated species in nondenaturing and denaturing gels with untreated M1 RNA and substrate, and (ii) by repeating the experiments with either ^{32}P-labeled M1 RNA or ^{32}P-labeled substrate and by following the appearance of label in the various bands shown in Fig. 2. In these experiments, radioactive label derived from both M1 RNA and substrate appeared only in bands d and d′.
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13. Reverse transcriptase can catalyze a non-templated, blunt-end addition (*12*), but that phenomenon is usually absent when nucleotides have been modified by chemical or physical agents. In these latter cases, the reverse transcriptase will undergo chain termination before the modified nucleotides, and these events are seen as intense bands in autoradiographs of the analyses of the reverse transcriptase reaction (*12*).
14. For hybridization, deoxyribonucleotide primers were labeled with [γ-^{32}P]ATP and polynucleotide kinase (Promega) as described by T. Maniatis, E. Fritsch, and J. Sambrook [*Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)]. Sequencing ladders, used as standards for the determination of the position of bands in the lanes that corresponded to the primer-extension reactions, were produced by the Sequenase system (U.S. Biochemical) from the plasmid DNA that contained the genes for M1 RNA or pTyr. The same primers as used in the extension reactions were used for the preparation of the sequencing ladders. To verify that the bands observed in the samples of enzyme-substrate complex were due to crosslinks between the two molecules, we performed primer extension reactions on M1 RNA in the presence or absence of substrate in equal molar amounts. Our results (*8*) indicate that the simple presence of free substrate in the M1 RNA sample does not generate the stops observed in the samples of enzyme-substrate complex. Similar results were obtained when M1 RNA was added to the primer extension reaction with the substrate.
15. For deletion of nucleotide C92 from M1 RNA, DNA from a clone that contained 87 bp from the 5′ terminal portion of the gene for M1 RNA (*8*) was digested with Fok I; the mixture was then ligated to an oligonucleotide that lacked nucleotide C92 of M1 RNA, and to a BstN I–Hind III fragment from plasmid pJA2′ (*3*), the source of the 3′ terminal portion of the gene for M1 RNA. The final ligated fragment was cloned between the EcoR I and Hind III sites of pUC19 (*3*). A Fok I digest of the resulting plasmid was used as a template for T7 RNA polymerase in vitro to generate Δ92 M1 RNA. The other mutant derivatives of M1 RNA were prepared and screened by techniques adapted from those of M. J. Zoller and M. Smith, *Nucleic Acids Res.* **10**, 6487 (1982) and W. Kramer *et al.*, *ibid.* **12**, 9441 (1984). The frequency of mutagenesis varied between 1 and 10 percent, depending on the particular mutagenic oligonucleotide. Preparation of M1 RNA transcripts in vitro was as described in (*3*).
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18. A derivative of pUC19 carrying the TyrT locus (7) was constructed with the sequence GGGATGCCATCCTGCAGGCATGCAAGCTT beyond the 3′ terminal CCA. This sequence contains sites recognized by BstN I, SfaN I, Fok I, Pst I and Hind III. The DNA was digested with BstN I, Fok I or SfaN I for use as a template for T7 RNA polymerase in vitro to generate pTyr − UCA, pTyr − CAUCA, and pTyr − CCAUCA, respectively (L. Kirsebom and C. Guerrier-Takada, unpublished).
19. The SupS1 plasmid, a gift from Ian Willis and D. Soll, Yale University [G. Krupp, B. Cherayil, D. Fendeway, S. Nishikawa, D. Soll, *EMBO J.* **5**, 1967 (1986)], was digested with Taq I prior to transcription in vitro with SP6 RNA polymerase.
20. The lengths of the 5′ terminal fragments of pTyr − CCAUCA, which resulted from cleavage by M1 RNA, Δ92 M1 RNA or by RNase P, made with either of the two RNA's, were determined from comparisons with the mobility of products of mild alkaline hydrolysis of end-labeled M1 RNA or pTyr − CCAUCA in polyacrylamide sequencing gels. The nature of the end groups produced was determined by extraction of the cleavage products from preparative polyacrylamide gels, digestion to completion with RNase T_2 (2.5 units of RNase T_2 in 50 m*M* sodium acetate, *p*H 5.5, overnight at 37°C), and two-dimensional thin-layer chromatography as described [C. Guerrier-Takada, A. van Belkum, C. W. A. Pleij, S. Altman, *Cell* **53**, 267 (1988)].
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