Model Substrates for an RNA Enzyme

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M1 RNA, the catalytic RNA subunit of *Escherichia coli* ribonuclease P, can cleave novel transfer RNA (tRNA) precursors that lack specific domains of the normal tRNA sequence. The smallest tRNA precursor that was cleaved efficiently retained only the domain of the amino acid acceptor stem and the T stem and loop. The importance of the 3′ terminal CCA nucleotide residues in the processing of both novel and normal tRNA precursors implies that the same enzymatic function of M1 RNA is involved.

MOLECULES OF TRANSFER RNA (tRNA) are synthesized as larger precursors that are enzymatically converted to mature tRNA in a series of reactions (*1*). As a key step in this process, ribonuclease (RNase) P cleaves the precursor to generate the 5′ terminus of the tRNA (*2*). A variety of experimental evidence has shown that RNase P can cleave many precursors, irrespective of different primary sequences, because the enzyme recognizes the common tRNA domain. This was first revealed by mutational studies in which an alteration to a single nucleotide involved in the secondary or tertiary structure of the precursor tRNA retarded cleavage (*3*). This relation of structure to substrate capacity prompts the question, what specific structure of the precursor is recognized by RNase P? The structural determinant could involve, for example, the entire tRNA domain or a smaller, specific part of it; alternatively, only the invariant bases distributed over the molecule may be involved.

RNase P is quite distinctive. The enzyme from *Escherichia coli* is a ribonucleoprotein (*4*) containing a catalytic RNA subunit (M1 RNA) and a protein subunit (C5 protein), both of which are essential for function in vivo (*5*). M1 RNA alone, or the RNase P holoenzyme, is capable of cleaving precursor molecules in vitro to generate the correct 5′ terminus of the tRNA. Since the Michaelis constant (K_m) of the reaction is the same with or without the protein subunit, it is likely that the RNA subunit alone is responsible for substrate recognition and cleavage, though the details of such a process are unknown (*5*). Enzymatic activities similar to RNase P have been found in extracts of other organisms (*6*). We have used oligonucleotide synthesis to create novel genes encoding precursors with specific domains of the tRNA. Some of these novel precursors were cleaved efficiently so that models of the RNase P substrate could be considerably simplified.

The nucleotide sequence of most tRNAs can be folded into a common secondary structure referred to as the cloverleaf. The common three-dimensional structure has two helical regions, one containing the acceptor stem and the T stem and loop, and the other, the D stem and loop and the anticodon stem and loop (Fig. 1) (*7*). Some mitochondrial tRNAs are atypical and lack the D stem and loop region of the cloverleaf. Despite this smaller size, the acceptor stem and the T stem and loop form a helix similar to the corresponding part of typical tRNAs (*8*). RNase P from *E. coli* should cleave this smaller precursor tRNA if it contains the critical substrate structure.

We synthesized complementary DNA fragments encoding an amber suppressor gene of *E. coli* $tRNA^{Phe}$ (*9*) but altered them to give a gene product with the length and three-dimensional structure of bovine mitochondrial $tRNA^{Ser}$ (*8*). Nucleotides in the D stem and loop were deleted, and sequences characteristic of the mitochondrial $tRNA^{Ser}$ were substituted. This gene derivative, termed TDF1 (Fig. 2), was inserted into Eco RI/Pst I sites of expression plasmid pGFIB (*10*) to facilitate measurement of RNA synthesis in vivo. Variants of TDF1 were made with less (TDF1-3) or different (TDF2 and TDF2-2) nucleotides in the T loop (Figs. 2 and 3). Northern gel analysis and analysis of the RNA labeled in vivo showed that tRNA-sized molecules were synthesized even though the suppressor genes were inactive (*11*). The various synthetic DNA fragments were recloned (*12*) into transcription plasmid pGEM-2 (*13*), and the resulting precursor was tested as a substrate for M1 RNA and the RNase P holoenzyme. All of the precursor substrates were cleaved by M1 RNA, though at different rates (Table 1). The presence of the C5 protein subunit increased the reaction rate but not as much as with a normal substrate, such as the precursor to $tRNA^{Phe}$. The nucleotide alterations in the T loop had only small effects on the rates. Similar experiments have been carried out with mitochondrial-like derivatives of *E. coli* $tRNA^{Gln}$ with essentially the same result (*11*).

We also assembled a synthetic derivative, termed AT1, that contained only the acceptor stem, the T stem and loop, and the 3′ terminal CCA nucleotide residues of the $tRNA^{Phe}$ gene; the remainder of the $tRNA^{Phe}$ structure was deleted (Figs. 2 and 3) (*12*). This precursor was readily cleaved by M1 RNA, and C5 protein did not greatly enhance the rate of cleavage (Table 1). The K_m and k_{cat} were very similar to values obtained with a standard precursor transcript containing the intact $tRNA^{Phe}$ sequence (Table 2). Mature AT1 RNA was sequenced by digestion with RNase T_1 (*9*), and the products were analyzed on a polyacrylamide sequencing gel. Comparison with RNase T_1 products from appropriate controls (AT1 precursor, $tRNA^{Phe}$ precursor, and $tRNA^{Phe}$) indicated that RNase P cleavage generated the 5′ terminus of the mature AT1 RNA (Fig. 3) and released the correct 5′ flanking oligonucleotide (*11*).

The secondary structure of AT1 and the related AT1-7 (see below) were investigated by 3′ end labeling with ^{32}P-pCp and partial digestion with cobra venom nuclease, which cleaves double-stranded regions preferentially (*14*), and with RNase T_1, which

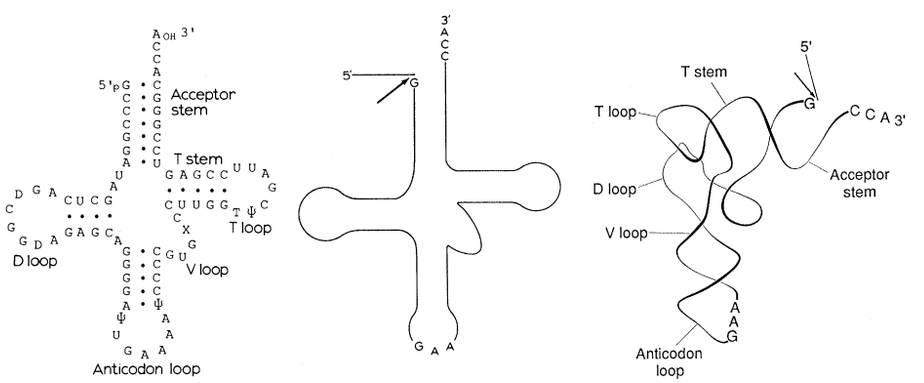

Fig. 1. Cloverleaf diagram of *E. coli* $tRNA^{Phe}$ (left panel) (*9*). The U at position 8 is modified, 4-thio-U, A at position 37 is methylthisopentenyl-A, and G at position 46 is 7-methyl-G in the mature RNA. Schematic versions of the phosphodiester backbone of this sequence as a precursor molecule shown in two dimensions (center panel) and three dimensions (right panel) (*7*). The arrow marks the site cleaved by RNase P.

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cleaves preferentially after Gp residues in single-stranded regions (*9*). We found that cobra venom nuclease attacked the double-stranded stem region, and that RNase T_1 attacked the single-stranded nucleotides located on the 5′ terminus and on the loop (Fig. 4). The digestion patterns of AT1 and AT1-7 precursors were indistinguishable except for the presence of the 3′ terminal GCAC in AT1-7. The secondary structure of the AT1 precursor shown in Fig. 4 is consistent with these results.

The novel size and shape of the AT1 precursor (Fig. 4) made it imperative to demonstrate that its cleavage requires the same enzymatic function of the M1 RNA and holoenzyme as in typical precursors. We noted that the 3′ terminal CCA sequence is a common feature of tRNAs (*1*). Furthermore, the 3′ terminal CCA sequence is found in all *E. coli* tRNA precursors and is important for efficient cleavage by RNase P and M1 RNA (*15, 16*). Therefore, derivatives of AT1 were assembled that altered the single-stranded 3′ terminal CCA nucleotide sequence to either GCA (AT1-7) or ACA (AT1-8) (Figs. 2 and 3) (*12*). Neither precursor was cleaved (Table 1) until the amount of RNase P holoenzyme was increased 20-fold, and then only about 4% of AT1-7 was processed. We emphasize that even though AT1, AT1-7, and AT1-8 precursors share identical primary sequences (except for the noted CCA sequence difference) and secondary structures (AT1-8 was not examined), a 3′ terminal CCA sequence on the precursor is required for efficient cleavage. Thus, a single function of the enzyme is indicated.

Two derivatives of the AT1 gene were assembled that altered the helix of the precursor. In derivative AT2, the T loop size was increased at the expense of the 5 bp on the T stem. In derivative Linear19, half of the nucleotides of AT1 were deleted to produce a linear transcript (Fig. 3). We have not analyzed the secondary structure of these precursors. Neither the AT2 nor the Linear19 precursor was cleaved under the conditions used (Table 2), showing that these alterations to the helix inactivated the substrate capacity of the precursors. No attempt was made to determine if cleavage would occur under conditions generally regarded as nonphysiological, such as in buffers containing very high salt concentrations or organic solvents (*5*).

The 4.5*S* RNA of *E. coli* is a small molecule that may be involved in protein synthesis, though not as a tRNA (*17*). A precursor

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TDF1:  5' AATTCGCCCGGATATGCGGGGATTCTAAATCCCCGCTCCTTGGTCTAATAGCCGAGTCCGGGCACCACTGCA 3'
          3' GCGGGCCTATACGCCCCTAAGATTTAGGGGCGAGGAACCAGATTAT'GGCTCAGGCCCGTGGTG 5'

TDF2:  5' AATTCGCCCGGATATGCGGGGATTCTAAATCCCCGCTCCTTGGTCTAATAGCCGAGTCCGGGCACCACTGCA 3'
          3' GCGGGCCTATACGCCCCTAAGATTTAGGGGCGAGGAACCAAGTTAW'GGCTCAGGCCCGTGGTG 5'

AT1:   5' AATTCGCCCGGACTCGGTTCGATTCCGAGTCCGGGCANCACTGCA 3'
          3' GCGGGCCTGAGCCAAGCTAAGGCTCAGGCCCGTGGTG 5'

AT2:   5' AATTCGCCCGGACTCGGTTCGATTCCGAGTCCGGGCANCACTGCA 3'
          3' GCGGGCCTGAGCCAAGCTAACCGAGAGGCCCGTGGTG 5'

Linear 19:        5' AATTCGCCCGGACTCGGTTCGATTCTGCA 3'
                     3' GCGGGCCTGAGCCAAGCTAAG 5'
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Fig. 2. Structure of synthetic oligonucleotides. A computer program aided the design (and later verification) of sequences by modifying *E. coli*–type tRNA sequences stored in a data bank. Each gene was derived from two synthetic oligonucleotides. The two oligonucleotides for a synthetic amber suppressor gene of *E. coli* $tRNA^{Phe}$ are not shown since a similar gene has been described (*10*). The synthetic oligonucleotides, when annealed, contained the entire gene for the mature RNA and flanking restriction-enzyme sites. Insertion of the oligonucleotides into a plasmid vector downstream from a promoter, and subsequent transcription, produced a precursor with 5′ leader sequences derived from the plasmid's promoter region and the gene's 5′ restriction-enzyme site. (Figure 3 shows the sequences of the mature RNAs in cloverleaf form.) The two oligonucleotides need not exhibit perfect complementarity because mismatches are corrected by repair and replication. In the case of a mismatch, recloning of plasmid DNA gave cells with homogeneous plasmid populations, and gene structure was established by DNA sequencing. The TDF2 construct was assembled from TDF1 (longer) plus TDF2 (shorter) oligonucleotides. The AT2 construct was assembled from AT1 (longer) plus AT2 (shorter) oligonucleotides. Nucleotide mixes were used at the indicated positions in TDF1, TDF2, and AT1 constructs; nucleotide abbreviations in these sequences are: ′ = deletion; W = A,T; and N = A,G,T,C. Oligonucleotides were synthesized on an Applied Biosystems model 380A DNA synthesizer (Biotechnology Center, University of Wisconsin), purified on a 12% (or 20% gel when the oligonucleotide was less than 40 residues in length) polyacrylamide-urea gel, and visualized by ultraviolet shadowing. They were excised, electroeluted, and 5′ end phosphorylated with kinase. The oligonucleotides were annealed over a 3-hour period to yield an insert fragment with 5′ Eco RI and 3′ Pst I restriction-enzyme sites. The insert fragment was annealed overnight to Eco RI/Pst I–digested pGFIB, and transformed into competent XAC/A16 cells (*lacpro, nalA, rif,* am*ArgE*/F′ *lacpro*X, am*Iq*-Z fusion) (*10*). Plasmid pGFIB contains an *AmpR* gene, an origin of phage f1, and a multiple cloning region (including restriction-enzyme sites for Eco RI and Pst I) flanked by a constitutive promoter and a terminator (*10*). The synthetic fragment was inserted into pGFIB such that transcription from the promoter produced tRNA or a comparable molecule with the sense of tRNA. Cells were plated on LB agar plus ampicillin. Colonies were lifted onto nitrocellulose disks and hybridized with 5′ end ^{32}P-phosphorylated oligonucleotide. Colonies with positive hybridization results were purified by streaking on LB agar plates plus ampicillin. Single-stranded DNA for dideoxy sequencing was obtained by inducing an origin of phage f1 on the pGFIB plasmid with phage IR1. A double-blind procedure was used to read the sequence gel; this involved four independent readings by two individuals using a computer program with a proofreading feature.

Table 1. Comparison of relative cleavage rates of M1 RNA and M1 RNA + C5 protein with different substrates. Determinations were made in the linear range of the kinetic curves (*5*) and normalized to the rate obtained with a precursor to *E. coli* $tRNA^{Tyr}$. The M1 RNA and reconstituted RNase P were prepared as described (*5*). The "p" in front of each substrate name defines them as precursors to molecules shown in Fig. 3 and to other substrates mentioned in the text. All measurements are reproducible within ±10%.

Substrate	Relative rates		Increase in rate
	M1 RNA	M1 RNA + C5 protein	
pTyr*	100	1100	11
pPhe	66	690	10.5
pTDF1	95	152	1.6
pTDF1-3	114	143	1.2
pTDF2	39	284	7.4
pTDF2-2	47	120	2.5
pAT1	99	349	3.5
p4.5*S*†	2	40	20
pAT1-7‡			
pAT1-8‡			
pAT2‡			
pLINEAR19‡			
pRCR‡	0	0	

*A precursor to *E. coli* $tRNA^{Tyr}$ made by in vitro transcription of a derivative of pGEM-1 (Promega Biotec) (*20*) and used as an independent standard for these measurements. †The precursor to 4.5*S* RNA was prepared as described (*5*). ‡These substrates gave no detectable cleavage (less than 1% of that seen with pPhe) under the conditions used. Substrate pRCR has the sequence: pppGAAUACACGGAAUUCGAGCUCGCCCCAUCGAUGGGGCGAGCUCGAAUUCUAG; the expected site of cleavage, if any, by RNase P is between G9 and G10 on the 5′ side of a hypothetical stem (18-bp) and loop (4-base) structure. The substrate was prepared as follows: An Eco RI (blunt-ended with Klenow)/Ava I fragment of pGEM-1 DNA (13 bp in length) was cloned into the Xba I (blunt-ended with Klenow)/Ava I site of pGEM-2 DNA (*13*). The resulting plasmid DNA was digested with Sma I, and an 8-bp Cla I linker was inserted. The DNA from the plasmid was digested with Xba I, and the resulting linear DNA was transcribed in vitro by SP6 RNA polymerase. This substrate forms a hairpin loop in which there are 17 bp starting with the nucleotide at position 10 from the 5′ end.

to 4.5*S* RNA is cleaved in vitro by RNase P (*18*) and M1 RNA (Table 1) but at considerably reduced rates. Interestingly, the sequence of this precursor can be arranged so that cleavage occurs on the 5′ side of a double-stranded segment, and the 3′ end of this double-stranded region contains a CCA sequence. The double-stranded segment is relatively long (39 bp) and contains 18 unpaired bases and a single-stranded loop of 6 bases. The analogy of such a structure to that of tRNA precursors was noted previously, and it was postulated that RNase P binds to its substrates at a double-stranded region (*18*). We have shown, however, that not all double-stranded RNAs are cleaved by M1 RNA or RNase P (for example, AT2 and pRCR) (Table 1). It remains to be determined what structural feature of the 4.5*S* RNA precursor makes it a substrate, albeit a poor one, for RNase P.

Table 2. Kinetic parameters of cleavage reactions.

Substrate	Enzyme	K_m (*M*)	k^*_{cat}
pPhe	M1	4.4×10^{-8}	0.34
	M1 + C5	4.2×10^{-8}	18.3
pTDF1-3	M1	1.2×10^{-7}	0.88
	M1 + C5	1.2×10^{-7}	9.9
pAT1	M1	2.0×10^{-8}	0.12
	M1 + C5	5.0×10^{-8}	8.2

*Moles of product per minute per mole of enzyme.

An unusual mutation of a phage suppressor gene has been found that involves the insertion of a 35-nucleotide duplication of the anticodon and V regions of the molecule. The mutation inactivates the suppressor function of the tRNA. Nevertheless, the mutated tRNA is synthesized in vivo, and RNase P cleaves the precursor in vitro. Nuclease digestion of the mutant tRNA suggests that the secondary structure of the acceptor and T stems is normal (*19*). The results with both *E. coli* 4.5*S* RNA precursor and the mutated phage tRNA precursor are consistent with the principal findings in this report.

We have shown that a small precursor bearing only the helical segment of the acceptor stem and T stem and loop retains the specificity and kinetic constants that RNase P exhibits for normal precursor substrates. We propose that this structure contains an important determinant for precursor recognition and cleavage. Catalysis with this molecule is similar to the known function of RNase P in that the requirement for the 3′ terminal CCA sequence is maintained. However, the 3′ terminal CCA assumes a greater importance for in vitro cleavage in this small precursor than in normal precursors (Table 1) (*5, 15*). RNase P might not cleave at a helical structure similar to that found in AT1 when it is present in the context of other sequences. This property may allow the enzyme to discriminate against RNAs with other metabolic fates.

As previously mentioned, base-substitution mutations in any part of the precursor tRNA moiety that have an impact on tRNA conformation reduce cleavage by RNase P (*3*), and yet, we have deleted roughly one-half of the tRNA sequence without affecting the rate or specificity of this cleavage. How can this apparent discrepancy be resolved? We suggest that base-substitution mutations affect precursor cleavage indirectly; either the mutated segment creates a structure that prevents the precursor from entering the catalytic site of the enzyme, or the important helical structure is denatured.

The next phase of this work will involve constructions of a family of variant precursors that retain the ability to act as substrates for RNase P. As more synthetic genes are designed and analyzed, the relationship between substrate structure and RNase P cleavage will be understood in greater detail.

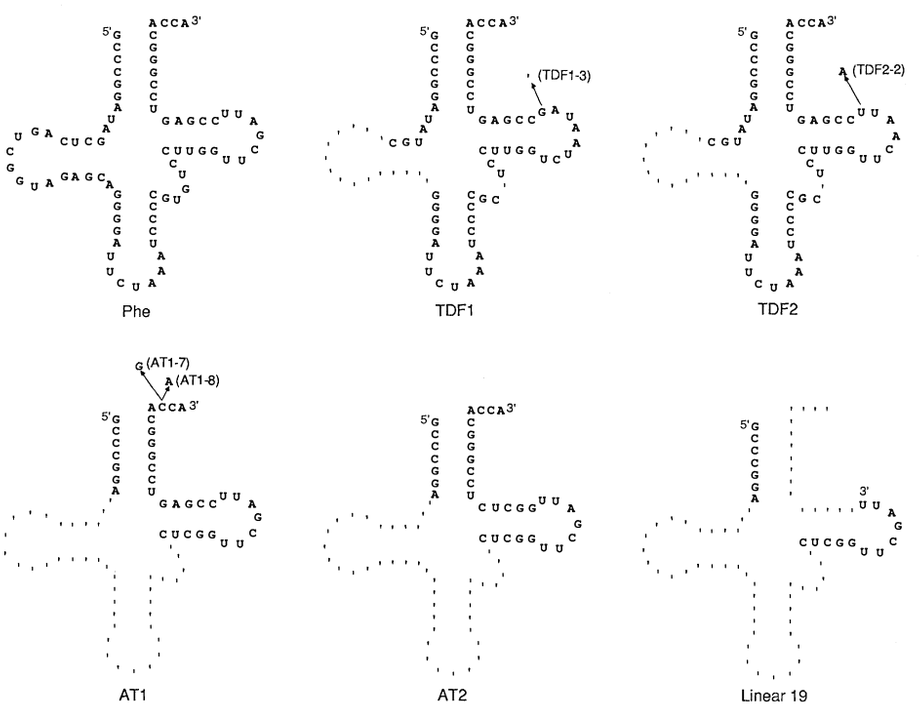

Fig. 3. Structure of mature RNAs encoded by synthetic genes. The sequences are shown without the modified nucleotides characteristic of the mature tRNA (Fig. 1) because they are not present in the transcripts made in vitro.

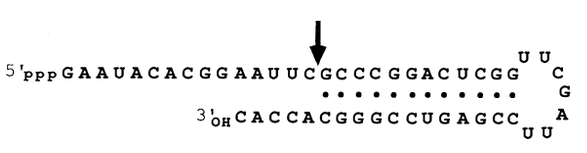

Fig. 4. Structure of the AT1 precursor drawn in a stem and loop structure similar to the corresponding region in $tRNA^{Phe}$. The arrow marks the internucleotide bond cleaved by RNase P. This precursor was synthesized in vitro from plasmid pGEM-2. The 5′ leader segment of the precursor was derived from the promoter region of plasmid pGEM-2 (*12*) and the Eco RI restriction-enzyme site of the gene. The 3′ terminal C was derived from the Pst I restriction-enzyme site of the gene. The sequence of the mature AT1 RNA is shown in Fig. 3.

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12. Synthetic genes in plasmid pGFIB flanked by Eco RI/Pst I restriction-enzyme sites (Fig. 2) were cloned into plasmid pGEM-2 (Promega Biotec) for in vitro transcription studies. The manufacturer's protocols were used. Briefly, plasmid pGFIB DNA was digested by Eco RI/Pst I, the fragment carrying the synthetic gene was isolated and inserted into the Eco RI/Pst I site of plasmid pGEM-2 DNA. Plasmid pGEM-2 carrying the synthetic gene was digested with Pst I, and the resulting linear DNA was transcribed in vitro by SP6 RNA polymerase. SP6 transcription of this plasmid gives a precursor containing a short 5′ leader sequence, pppGAAUACACGGAAUUC (*13*), and an extra 3′ C residue corresponding to the residual part of the Pst I–digested restriction-enzyme site.
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Recombinant Fragment of Protein Kinase Inhibitor Blocks Cyclic AMP–Dependent Gene Transcription

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Transcriptional regulation by cyclic adenosine monophosphate (cAMP) in mammalian cells could be mediated by a phosphoprotein substrate of the cAMP-dependent protein kinase or, as in prokaryotes, by a cAMP-binding protein. Two synthetic genes that code for an active fragment of the protein inhibitor of this kinase and a mutant inactive fragment were constructed and used to distinguish these alternatives. Transient expression of the active peptide product specifically inhibited the cAMP-stimulated expression of a cotransfected reporter gene by more than 90 percent, whereas the expression of the inactive peptide did not alter cAMP-stimulated gene expression. The results indicate that an active kinase catalytic subunit is a necessary intermediate in the cAMP stimulation of gene transcription.

Protein kinases and protein phosphatases catalyze a set of reversible posttranslational modifications that are associated with the regulation of many cellular processes (*1, 2*). Certain of these enzymes are subject to inhibition by high-affinity, specific interaction with other proteins, such as the heat-stable inhibitor of the cyclic adenosine monophosphate (cAMP)–dependent protein kinase (PKI, protein kinase inhibitor), first described by Walsh and his colleagues (*3*); PKI is a high-affinity (K_i near 1 n*M*) inhibitor that competes with protein substrates for binding to the free catalytic subunit of the kinase (*3*). Studies with synthetic peptides corresponding to the amino acid sequence of rabbit skeletal muscle PKI have demonstrated the importance of the pseudosubstrate site RRNAI (*4*) (residues 18–22), as well as residues nearer the amino terminus, in conferring the very high affinity and specificity of PKI (*5*). Thus most, perhaps all, of the structural features required for kinase inhibition are located within the NH_2-terminal third of rabbit skeletal muscle PKI, a 75–amino acid protein.

We synthesized a peptide [PKI(1–31), Fig. 1A] corresponding to the NH_2-terminal 31 residues of the rabbit skeletal muscle PKI (*6*). This peptide inhibited competitively (K_i = 4 nM) the phosphorylation of a synthetic peptide substrate (LRRASLG) by the catalytic subunit of cAMP-dependent protein kinase. The specificity of purified PKI(1–75) was maintained by synthetic PKI(1–31). In assays in vitro with purified enzymes, the cyclic guanosine monophosphate–dependent protein kinase, protein kinase C, and protein phosphatase 1 were not inhibited at all by PKI(1–31) at concentrations up to 1.9, 6.1, and 12 μ*M* peptide, respectively. The crucial role of PKI residues 18 and 19 was confirmed by synthesis of PKI peptides in which one or both of these arginine residues had been replaced by glycine; these peptides exhibited greatly diminished ([Gly^{19}]PKI(1–31)) or no detectable PKI activity ([$Gly^{18,19}$]PKI(1–31), Fig. 1B).

A gene coding for an initiator methionine and PKI(1–31) was designed and constructed from synthetic oligonucleotides (*7*) (Fig. 1A); a gene coding for the inactive peptide [$Gly^{18,19}$]PKI(1–31) was prepared by replacing the Sfi I–Bsm I restriction fragment of the PKI(1–31) minigene with mutant oligonucleotides that would replace arginines 18 and 19 in the PKI sequence with glycines. Wild-type PKI(1–31) and mutant [$Gly^{18,19}$]PKI(1–31) peptides were expressed in *Escherichia coli* as lacZ fusion proteins; insoluble inclusion bodies of the parent lacZ product and the fusion proteins were partially purified, treated with CNBr, lyophilized, and reconstituted in neutral buffer. The mixture of CNBr peptides was assayed for PKI content by radioimmunoassay and for protein kinase inhibitory activity. Whereas the CNBr peptides derived from the lacZ-[$Gly^{18,19}$]PKI(1–31) fusion product showed substantial PKI-like immunoreactivity, this preparation gave no inhibition of protein kinase activity (Fig. 1B). In contrast, the CNBr peptides derived from lacZ-PKI(1–31) fusion protein exhibited protein kinase inhibitory activity, which, in relation to the measured content of PKI(1–31), had a comparable or slightly greater potency than that of the synthetic PKI(1–31) peptide (Fig. 1B). Thus the synthetic minigene for PKI(1–31) codes for a functional PKI(1–31) peptide, whereas the mutant minigene [$Gly^{18,19}$]PKI(1–31) is itself functional but codes for a peptide that lacks kinase inhibitory activity.

The synthetic genes were cloned into the eukaryotic expression vector πLXX (*8*) to produce the plasmids πLXX-PKI(1–31) (Fig. 1C) and πLXX-[$Gly^{18,19}$]PKI(1–31). Expression of πLXX-PKI(1–31) was examined after transfection of CV-1 and COS-M6 cells. Hybridization of total RNA with PKI ^{32}P-labeled oligonucleotides revealed a 1.2-kb RNA species in both cell lines, in agreement with the size predicted. S1 nuclease protection assay with a uniformly labeled RNA probe that recognized both πLXX and πLXX-PKI(1–31) messenger RNAs (mRNAs) gave protected fragments of the expected size. Neither the size nor abundance of πLXX or πLXX-PKI(1–31) mRNA was affected by cAMP analogs.

Translation of PKI(1–31) mRNA to produce the recombinant peptide was demonstrated after transfection of COS-M6 cells. Extracts of cells transfected with πLXX or

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