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Evolutionary Origins of *apoB* mRNA Editing: Catalysis by a Cytidine Deaminase That Has Acquired a Novel RNA-Binding Motif at Its Active Site

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Summary

The site-specific C to U editing of apolipoprotein B100 (*apoB100*) mRNA requires a 27 kDa protein (p27) with homology to cytidine deaminase. Here, we show that p27 is a zinc-containing deaminase, which operates catalytically like the E. coli enzyme that acts on monomeric substrate. In contrast with the bacterial enzyme that does not bind RNA, p27 interacts with its polymeric *apoB* mRNA substrate at AU sequences adjacent to the editing site. This interaction is necessary for editing. RNA binding is mediated through amino acid residues involved in zinc coordination, in proton shuttling, and in forming the αβα structure that encompasses the active site. However, certain mutations that inactivate the enzyme do not affect RNA binding. Thus, RNA binding does not require a catalytically active site. The acquisition of polymeric substrate binding provides a route for the evolution of this editing enzyme from one that acts on monomeric substrates.

Introduction

C to U (and in some cases U to C) editing is extensively documented in the mitochondria and chloroplasts of vascular plants (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1994). The conversion of multiple C to U residues creates mRNAs that can be translated into proteins corresponding to those that are genomically encoded by the mitochondrial genomes of species, in which editing does not take place. Apolipoprotein B (*apoB*) mRNA undergoes a much more discrete and specific C to U editing in the nucleus of certain mammalian cell types to generate a stop translation codon and a truncated form of apoB called apoB48 (241 kDa) because it is 48% of the size of the largest form of apoB, designated apoB100 (Chen et al., 1987; Powell et al., 1987). ApoB48 is made in the enterocytes of the small intestine and is required for the transport through the blood of dietary lipid in triglyceride-rich lipoproteins called chylomicrons. ApoB100 (512 kDa) is needed for the transport of hepatically synthesized lipids and is the sole protein of plasma low density lipoprotein.

The mechanism of *apoB* mRNA editing is most plausibly a sequence-specific cytidine deamination (Bostrom et al., 1990; Hodges et al., 1991). The catalytic subunit of the *apoB* mRNA–editing enzyme is a 27 kDa (p27) member of the cytosine nucleoside/nucleotide deaminase family of enzymes (Navaratnam et al., 1993a; Teng et al., 1993). p27 alone is not competent for *apoB* mRNA editing, but requires other cellular factors for RNA targeting. A candidate involved in RNA targeting is a protein of 60 kDa (p60) that can be specifically ultraviolet (UV) cross-linked to a sequence downstream of the editing site at C6666 in *apoB* mRNA (Navaratnam et al., 1993b). Yet, other cellular factors may also be involved. Editing has been proposed to be mediated by a large editosomal (as in spliceosomal) complex that assembles on *apoB* mRNA (Harris et al., 1993). It is also evident that the other factors required for editing are expressed in liver cells that normally lack this function, as mixing of extracts from human liver cells with p27, transfection of liver cell lines with p27, or transduction of rabbit liver with p27 is sufficient to produce site-specific editing of *apoB* mRNA (Teng et al., 1993; Giannoni et al., 1994; Hughes et al., 1994).

In considering the origin of *apoB*-type mRNA editing, it is important to understand how a cytidine deaminase that acts on monomeric substrates might have evolved to modify cytidine specifically in an oligoribonucleotide context. A priori, this might have occurred by mutation of a cytidine deaminase that acts on mononucleotides/nucleosides so that it binds to the RNA, or by the association of such a deaminase with an RNA targeting subunit(s), or by a combination of both. In the present study, we show that the catalytic subunit of the *apoB* RNA–editing enzyme is a zinc-containing cytidine residue deaminase, that it has acquired the ability to bind to *apoB* mRNA through its active site and adjacent residues, and that this RNA binding is necessary for *apoB* mRNA editing.

Results

Phylogenetic Relationships of the 27 kDa Catalytic Subunit of the mRNA Editing Enzyme

We have previously demonstrated that the catalytic subunit (p27) of the *apoB* mRNA–editing enzyme is related to the cytidine deaminase family of proteins (Navaratnam et al., 1993a). The crystal structure of the Escherichia coli cytidine deaminase has recently been established, and structural similarities with p27 have been noted (Betts et al., 1994). To help identify the structural motifs in p27 that are important for site-specific cytidine deamination in an oligoribonucleotide context, we have identified cDNA clones for the human enzyme and compared the sequence of this with the rat RNA-editing cytidine deaminase and with other deaminases at the proposed active site of the enzymes (Figure 1A). Analysis of the amino acid sequence at the active sites of the deaminases (Figure 1B) indicates that the CMP/dCMP deaminases and cytidine/deoxycytidine deaminases form phylogenetically distinct subgroups based on sequence homology. This classification is con-

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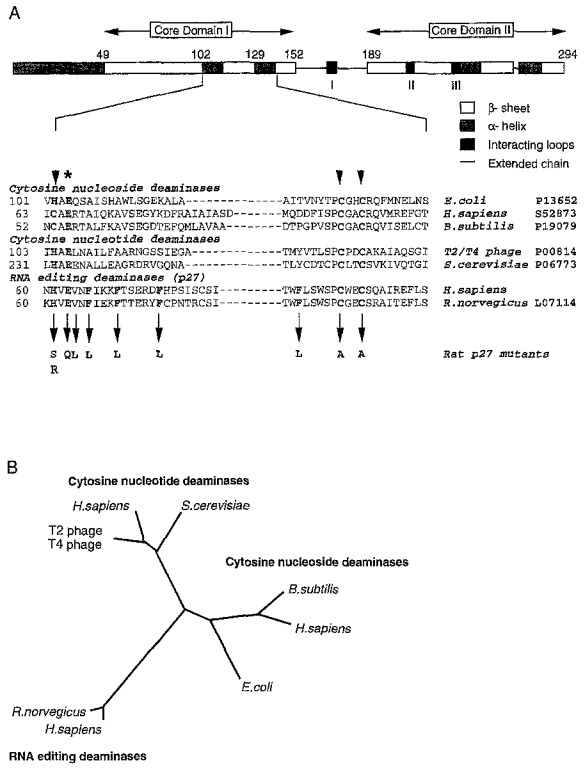

Figure 1. Domain Organization and Phylogenetic Analysis of the Active Site Regions of RNA-Editing Deaminases and Cytidine Deaminases

(A) Domain organization of E. coli cytidine deaminase, alignment of the active sites in the cytosine nucleoside/nucleotide deaminases and RNA-editing deaminases, and site-directed mutants of the rat RNA-editing deaminase. The E. coli cytidine deaminase (Betts et al., 1994) has two core domains connected by an extended chain. Core domain I contains the active site, zinc-liganding residues (arrows), and the Glu-104 residue (asterisk) that has the proton shuttle functions. In the dimeric enzyme, core domain II covers the active site cleft of the other monomer. The site-directed mutants were designed to minimize the disturbance to protein secondary structure.

(B) Phylogenetic analysis of the active site of cytosine nucleoside/nucleotide deaminases and the RNA-editing deaminases. A distance matrix was constructed from the alignment of the active site (residues 60–115 of rat p27) using the program PRODIST, and the phylogenetic tree was generated using the program KITSCH (PHYLIP version 3.5c). The tree indicates that the active sites of these three types of deaminases has evolved from a common ancestor to achieve the distinct substrate specificities observed.

cordant with the absence of overlap of substrate specificity between these two groups of deaminases that act on monomeric substrate (Ipata and Cercignani, 1978; Maley, 1978; Neuhard, 1978; Rothman et al., 1978; Wentworth and Wolfenden, 1978). The RNA-editing cytidine deaminases form a third distinct subgroup.

The deaminases all contain a conserved triad of histidine (or rarely cysteine), two paired cysteine residues that are proposed to coordinate zinc, and a glutamate residue proposed to act in a proton shuttle during deamination (Figure 1A). Additional residues conserved between the human and rat editing enzymes, but not found in any other member of the deaminase family, are four phenylalanines that reside in the segment that links the proposed active site residues.

Site-Directed Mutagenesis of p27 Identifies the Active Site

To evaluate the structure and function of the catalytic subunit of the *apoB*-editing enzyme (p27), we have expressed it as a glutathione S-transferase (GST) fusion protein in E. coli (GST–p27), and as an influenza haemagglutinin-tagged protein (HA–p27) in Sf9 insect cells. Neither GST–p27 nor HA–p27 was alone competent for editing. p27 was only active in the *apoB* mRNA editing assay, when complemented with partially purified rat intestinal extracts that had lost editing activity, presumably through loss of intrinsic p27. Surprisingly, we found that HeLa cell nuclear extract could also complement p27 (data not shown), even though HeLa cells express neither p27 nor *apoB*. Previously, we have shown that p27 expressed in Xenopus laevis has cytidine deaminase activity (Navaratnam et al., 1993a). However, neither the E. coli fusion protein nor the baculovirus form has this activity.

The homology of p27 to other deaminases has indicated residues that encompass the presumed active site (Figure 1A) (Navaratnam et al., 1993a; Bhattacharya et al., 1994). The crystal structure of the E. coli cytidine deaminase complexed to the transitional state analog 5-fluoropyrimidine-2-1 riboside indicates that the residues His-102, Cys-129, and Cys-132 are zinc-coordinating ligands and form the active site, together with the Glu-104, which is involved in proton transfer functions (Betts et al., 1994). These residues correspond to His-61, Cys-93, Cys-96, and Glu-63 in rat p27 (Figure 1A) and are conserved in the cytosine nucleoside/nucleotide deaminase family.

To establish whether these conserved amino acid residues form the active site of p27, site-directed mutagenesis of GST–p27 was performed to introduce "safe" changes at these residues (Bordo and Argos, 1991; Figure 1A). Replacement of His-61 by Ser (H61S) or Arg (H61R), Cys-93 and Cys-96 by Ala (C93A and C96A), and Glu-63 by Gln (E63Q) abolished editing activity (Figure 2A). Double mutants of Cys-93 and Cys-96 (C93A and C96A combined) and deletion of amino acid residues 93–96 also abolished editing (data not shown). Mutation of the residue Val-64, which is not conserved between deaminases, to Leu (V64L) had no effect on editing activity (Figure 2A).

p27 is a Zinc-Dependent Cytidine Residue Deaminase

To investigate the proposal that p27 is a zinc-dependent cytidine residue deaminase, we have used zinc-specific chelation and site-directed mutagenesis. In the first instance, HA–p27 expressed in Sf9 cells was incubated with a zinc-specific chelating agent, 1,10-o-phenanthroline. This abolished the ability of p27 to confer editing activity on partially purified rat intestinal extract that has lost this activity (Figure 2B). Control incubations with either 1,7-o-phenanthroline (Figure 2B) or with the ethanol vehicle (data not shown) used for solubilizing this compound had no effect. Removal of 1,10-o-phenanthroline and replacement of zinc by dialysis fully restored editing activity to HA–

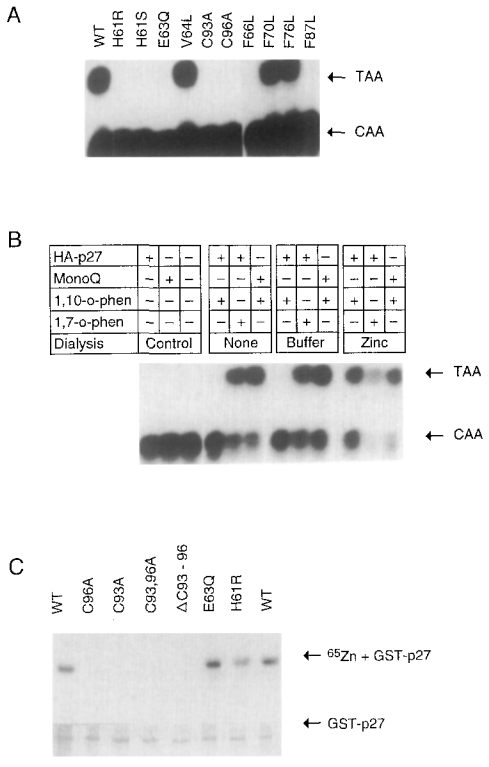

Figure 2. Mutational Analysis of the Active Site and RNA-Binding Sites of p27

(A) Effect of catalytic and RNA-binding site mutations on *apoB* mRNA editing. Mutation shown is of the zinc-binding ligands (H61R, H61S, C93A, and C96A), the glutamate involved in proton shuttling (E63Q), a neutral V64L change, and phenylalanine mutants F66L, F70L, F77L, and F87L. GST–p27 (WT) and the mutants (1 μg) were supplemented with Mono Q fraction (0.3 μg) and assayed for editing activity.

(B) p27 requires zinc for editing. HA–p27 and Mono Q fractions were incubated with 1,10- and 1,7-o-phenanthroline for 10 min. Aliquots of HA–p27 (1 μg) and Mono Q fractions (0.3 μg) were removed, and editing activity was assayed by supplementing with untreated Mono Q (0.3 μg) and HA–p27 (1 μg), respectively (none). The remaining mixtures were dialyzed overnight, and similar aliquots were removed and assayed for editing activity as before (buffer). The remainder of the mixtures were dialyzed in the presence of zinc, and the editing activity was assayed (zinc).

(C) Zinc binding by wild-type and mutant p27. GST-p27 (WT) and mutant (C96A, C93A, C93A and C96A combined [C93,96A], deletion of Cys-93 to Cys-96 [ΔC93–96], E63Q, and H61R) GST fusion proteins were cleaved with thrombin, separated by SDS–PAGE, and transferred to nitrocellulose membranes. Denatured membrane-bound proteins were renatured in the presence of ^{65}Zn and autoradiographed. Coomassie blue–stained wild-type and mutant GST–p27 are shown.

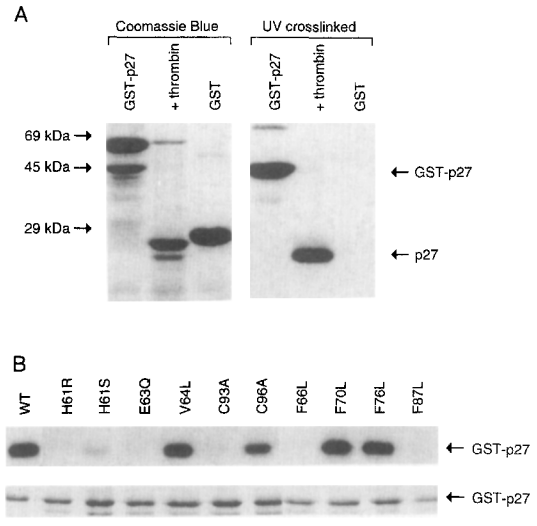

Figure 3. RNA Editing Deaminase Binding to *apoB* mRNA

(A) p27 UV cross-links to *apoB* mRNA. GST–p27 (1 μg) or GST–p27 (1 μg) after cleavage with thrombin, or GST alone was UV cross-linked to *apoB* RNA (wild-type A–I in Figure 4A). Cross-linked proteins were separated on 12% SDS–polyacrylamide gels and stained with Coomassie blue, and the autoradiograph of the same gel is shown. p27 did not UV cross-link to actin mRNA (Figure 4B), or to globin mRNA or antisense wild-type A–I transcript (data not shown).

(B) UV cross-linking of wild-type and mutant p27 to *apoB* mRNA. GST-p27 (WT) and mutant (H61R, H61S, E63Q, V64L, C93A, C96A, F66L, F70L, F77L, and F78L). GST–p27 was UV cross-linked to ^{32}P-labeled RNA and separated on 10% SDS–polyacrylamide gels. Coomassie-stained wild-type and mutant GST-p27 are shown.

p27. 1,10-o-phenanthroline had no effect on the activity of the complementing fractions of the editing enzyme, indicating that zinc was not necessary for activity of the complementing fraction.

To evaluate the role of p27 residues His-61, Cys-93, and Cys-96 in zinc coordinating, mutants in which these residues were altered were studied for their ability to bind zinc by probing with $^{65}Zn(II)$. Mutations C93A, C96A, C93A and C96A combined, and deletion of Cys-93 to Cys-96 reduced zinc binding to background levels (Figure 2C). Wild type and H61R, E63Q (Figure 2C), H61S, and V64L (data not shown) mutants each bound zinc. These results are in accord with the crystal structure of E. coli cytidine deaminase and with mutagenesis of the E. coli enzyme (Betts et al., 1994; Smith et al., 1994). In the E. coli cytidine deaminase, zinc is coordinated by His-102, Cys-129, and Cys-132. The E. coli mutants of Cys-129 and Cys-132 no longer bind zinc, but mutants of His-102 still show significant zinc binding. Thus, in the E. coli cytidine deaminase mutants and p27 mutants that alter the histidine, shown in the crystal structure of the E. coli enzyme to be involved in zinc coordination, zinc is still bound, presumably by the two cysteine ligands. p27 is therefore a zinc-containing cytidine deaminase with significant active site similarities to the E. coli enzyme.

p27 Binds *apoB* mRNA through Residues Encompassing the Active Site

p27 was evaluated for its ability to interact with *apoB* mRNA by UV cross-linking. Specific cross-linking of p27 to its RNA substrate (Figures 3A and 3B) was demonstrated. p27 did not UV cross-link to actin mRNA (see legend to Figure 3A). GST alone (Figure 3A) and the E. coli cytidine deaminase (data not shown) do not UV cross-link to *apoB* mRNA. As was previously demonstrated, UV cross-linking of active editing extract did not identify p27, probably because of low abundance (data not shown; Navaratnam et al., 1993b).

To ascertain the amino acid residues involved in RNA–protein interaction, mutants of the active site residues and

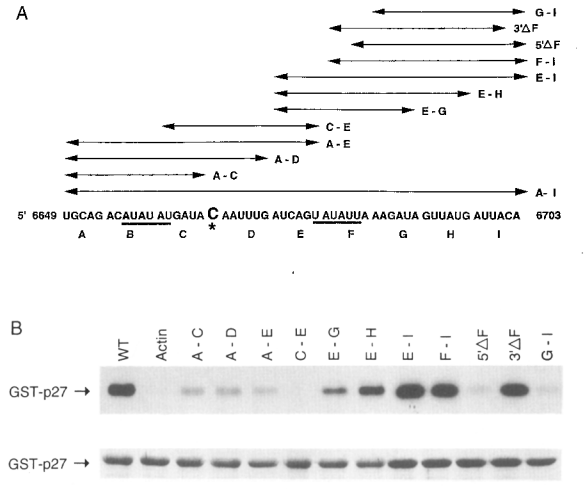

Figure 4. Mapping of RNA-Editing Deaminase–Binding Sites on *apoB* mRNA

(A) Wild-type and deletion mutants of *apoB* mRNA. Wild-type (A–I) *apoB* mRNA and deletion (A–C, A–D, A–E, C–E, E–G, E–H, E–I, F–I, 5′ΔF, 3′ΔF, and G–I) mutants of *apoB* RNA were prepared as ^{32}P-labeled substrates by T7 promoter–mediated in vitro transcription reactions. C6666 is marked by an asterisk. AU-rich sequences are underlined.

(B) UV cross-linking of GST–p27 to *apoB* RNA. GST–p27 (1 μg) was UV cross-linked to wild-type (WT) and deletion mutant (Figure 4A) *apoB* mRNA. Coomassie blue-stained wild-type GST–p27 are shown.

of other residues encompassing the active site were studied. Certain mutants in the catalytic domain of p27 that abolish editing (H61S, H61R, E63Q, and C93A) (Figure 3B), double-mutant C93A and C96A, and deletion of Cys-93 to Cys-96 (data not shown) also did not UV cross-link to the RNA substrate. The neutral mutant V64L did not affect UV cross-linking. The mutant C96A that abolishes editing and zinc binding showed only partially reduced UV cross-linking to *apoB* mRNA (Figure 3B).

A series of phenylalanine residues are conserved between rat and human p27 at positions 66, 70, 76, and 87. In no other deaminases are these residues conserved (Figure 1A). Phe-66 resides in the first active site α helix, and Phe-70, Phe-76, and Phe-87 reside in the β sheet that links the two active site α helices (Figure 1A). Mutation of residues Phe-66 (F66L) and Phe-87 (F87L) abolished editing, but mutation of Phe-70 (F70L) and Phe-76 (F76L) had no effect on editing (Figure 2A). F66L, F70L, F76L, and F87L each bound zinc to the same extent as wild type (data not shown).

We considered whether Phe-66 and Phe-87 might be involved in RNA binding and for positioning p27 for site-directed deamination of a C6666. In a variety of RNA-binding proteins in which RNA–protein interaction can be demonstrated by UV cross-linking, this occurs through free radical–driven covalent linkage to aromatic residues. The mutants F66L and F87L that do not edit also did not UV cross-link to substrate RNA (Figure 3B). Cytidine deaminase activity against monomeric substrate was not restored in these mutants (method as in Navaratnam et al., 1993a; data not shown). Mutants F70L and F76L had no effect on either editing or cross-linking. These results indicate that p27 interacts with *apoB* mRNA through certain active site residues in a highly specific manner. Phe-66 and Phe-87 are also involved in this interaction, as mutation of these residues abolished editing and UV cross-linking. Therefore, RNA binding by p27 is a prerequisite for *apoB* mRNA editing, but RNA editing and UV cross-linking can be separated by mutation, as demonstrated by the C96A mutant that fails to edit and bind zinc, but binds *apoB* mRNA. A functional active site is not therefore necessary for RNA binding.

Mapping of p27 Binding to *apoB* RNA

To define the RNA-binding site of p27, we established a series of deletion mutants encompassing the *apoB* mRNA–editing and cross-linking substrate (referred to as wild-type A–I, nucleotides 6649–6703) (Figures 4A and 4B). UV cross-linking to GST–p27 is present in deletions containing either the AU-rich hexanucleotide segment 6656–6661 (B–C), or the AU-rich segment 6678–6683 (E–F), and is lost in deletions lacking both these segments. This maps the binding site of GST–p27 to these two AU-rich segments. Experiments with other mutants (A–H, A–G, A–F, B–I, C–I, D–I, B–E, and C–F) were also performed (data not shown). These were shown to cross-link to GST–p27, confirming the above mapping data. The AU sequences in mutants containing the F segment cross-linked better than those containing only the B segment. The presence of both AU-rich sequences did not enhance UV cross-linking compared with mutants with a single AU-rich sequence (compare wild-type A–I with E–I in Figure 4B). Actin mRNA did not UV cross-link.

Previously, we and others have shown that nucleotides present in B–C can be deleted without abolishing editing and that conversion of the segment B to its complement and certain point mutations in this region enhance rather than reduce editing (Backus and Smith, 1991; Shah et al., 1991). In contrast, mutations of F to its complement and point mutation of this sequence substantially reduce or abolish editing. Together, these results suggest that p27 binding to the region F is important for editing. We therefore generated 5′ΔF and 3′ΔF, each of 21 nucleotides, by the deletion of three 5′ or 3′ nucleotides, respectively (Figure 4A). The mutant 5′ΔF showed weak UV cross-linking, whereas 3′ΔF UV cross-linked to p27 comparably to F–I and wild-type A–I, and better than A–C, A–D, A–E, E–H, and E–G. These results indicate that the segment E–F (nucleotides 6678–6683) contains the critical nucleotides for p27 binding, but that the AUU sequence in the segment I may contribute to UV cross-linking.

We considered that p27 might preferentially bind AU-containing RNA sequences, because of nucleotide content rather than sequence. To test this, the effect of competition with polyadenylic acid (poly[A]), polycytidylic acid (poly[C]), polyguanidylic acid (poly[G]), polyuridylic acid (poly[U]), polycytidylic and polyguanidylic acid double-stranded homopolymer (poly[C]·[G]), polyadenylic and polyuridylic acid double-stranded homopolymer (poly[A]·[U]), polyadenylic-polyuridylic-polyuridylic acid triple-stranded

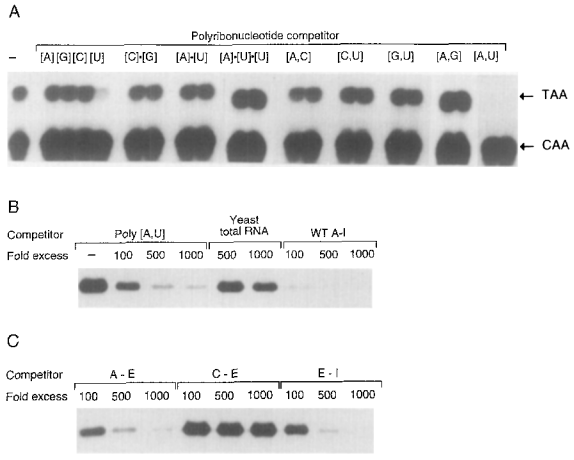

Figure 5. Effect of Synthetic Polyribonucleotides on p27 *apoB* mRNA Editing and Binding

(A) Effect of synthetic polyribonucleotides on *apoB* mRNA editing. GST–p27 (1 μg) was supplemented with Mono Q (0.3 μg) fractions in the absence (minus) and presence of indicated polyribonucleotides (polyadenylic acid, [A]; polyguanidylic acid, [G]; polycytidylic acid, [C]; polyuridylic acid, [U]; polycytidylic and polyguanidylic acid, [C]·[G]; polyadenylic and polyuridylic acid, [A]·[U]; polyadenylic-polyuridylic-polyuridylic acid [A]·[U]·[U]; polyadenylic and cytidylic acid [A,C]; polycytidylic and uridylic acid [C,U]; polyguanidylic and uridylic acid [G,U]; polyadenylic and guanidylic acid [A,G]; polyadenylic and uridylic acid [A,U]; 300-fold excess of [A],[G],[C],[U], 150- and 300-fold excess of other polyribonucleotides) and was assayed for editing activity. Editing reactions were set up as described.

(B) Effect of polyribonucleotide AU RNA on UV cross-linking. UV cross-linking of GST–p27 (1 μg) to wild-type (WT) A–I RNA was competed with the indicated fold excess of competitor (poly [A,U], yeast total RNA, WT A–I) over labeled substrate.

(C) Effect of deletion mutants on UV cross-linking. UV cross-linking of GST-p27 (1 μg) to WT A–I was competed with the indicated fold excess of competitor (A–E, C–E, and E–I) over labeled substrate.

homopolymer (poly[A]·[U]·[U]), polyadenylic and cytidylic acid (poly[A,C]), polycytidylic and uridylic acid (poly[C,U]), polyguanidylic and uridylic acid (poly[G,U]), polyadenylic and guanidylic acid (poly[A,G]), and polyadenylic and uridylic acid (poly[A,U]) on editing activity and on UV cross-linking was examined (Figure 5). Poly[A,U] RNA completely abolished editing in a concentration-dependent manner (Figure 5A). Poly(U) competition also reduced levels of editing. Other polynucleotides had little or no effect. p27 showed strong affinity for poly(A,U) RNA in UV cross-linking studies (Figure 5B), but little or no affinity for other polyribonucleotides (data not shown) or control yeast total RNA. Competition for the UV cross-linking of p27 to the segment wild-type A–I was also performed with segments A–E, C–E, and E–I. A–E and E–I both contain AU-rich sequences and were effective competitors, with E–I being better than A–E. C–E did not compete. We suggest that p27 has affinity for AU-rich sequences and binds these sequences both upstream and downstream of the editing site, but that the latter is the necessary binding for editing to occur.

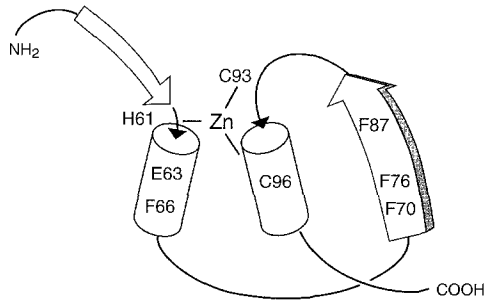

Figure 6. Schematic Representation of the Active Site of the RNA-Editing Cytidine Deaminase

The diagram shows zinc coordinated by His-61 (H61), Cys-93 (C93), and Cys-96 (C96) and the proton-shuttling glutamate Glu-63 (E63). Conserved phenylalanines Phe-66 (F66), Phe-70 (F70), Phe-76 (F76), and Phe-87 (F87) are shown in the linking β segment between the active site α helices. RNA binding is mediated by His-61, Glu-63, Phe-66, Phe-87, and Cys-93. This diagram is based on a cartoon in Betts et al. (1994).

Discussion

The present study establishes that the catalytic subunit of the *apoB* mRNA–editing enzyme is a member of the zinc-containing cytidine deaminase family of enzymes and that the tertiary structure of E. coli cytidine deaminase active site is conserved in the editing enzymes (Betts et al., 1994). In distinction from the E. coli enzyme, the editing enzymes form a phylogenetically separate subgroup of enzymes that have evolved the capacity to deaminate cytidine in an oligonucleotide context through binding *apoB* mRNA by residues used for zinc binding, proton shuttling, and forming the αβα structure that encompasses the active residues of the enzyme (Figures 1 and 6).

We and others have previously shown that the editing activity of rat intestinal extracts is dependent on zinc (Navaratnam et al., 1993a; Barnes and Smith, 1994). In the present study, we have shown that chelation with 1,10-o-phenanthroline specifically inhibits the activity of expressed p27. Replacement of zinc by dialysis reactivates the enzyme, demonstrating that the activity of expressed p27 requires zinc. The partially purified p27 complementing activity needed for editing from rat small intestine or heterologous complementing activity from HeLa cell splicing extracts is not inhibited by 1,10-o-phenanthroline and is therefore not zinc dependent. Thus, the zinc-dependent component of the editing activity is the p27 catalytic subunit of the *apoB* mRNA–editing complex.

Zinc is required for the activity of related deaminases, such as bacteriophage T4 dCMP deaminase and adenosine deaminase, and is present at the active site of E. coli cytidine deaminase (Wilson et al., 1991; Moore et al., 1993; Betts et al., 1994). The recently determined crystal structure of E. coli cytidine deaminase indicates that the active site of this enzyme is formed by an αβα motif (Figures 1 and 6), which contains the three zinc-coordinating residues His-102, Cys-129, and Cys-132 and the proton-shuttling residue Glu-104. The C4 of cytidine undergoes

a nucleophilic attack by the zinc-bound hydroxide. The Glu-104 residue has three roles: activation of the zinc-bound water, protonation of the N3 on cytidine, and shuttling of the proton from the zinc-bound hydroxide to the leaving ammonia. This results in the conversion of cytidine to uracil with the production of ammonia. The above residues are conserved in other cytosine nucleoside/nucleotide deaminases, and in p27, in which presumably they also form the active site.

We introduced "safe" mutations at the potential zinc-binding amino acids in p27 (H61R, H61S, C93A, and C96A) by site-directed mutagenesis (Figures 1 and 6). The mutations were based on the preferred amino acid substitution matrix and are least likely to disturb protein secondary structure (Bordo and Argos, 1991). We also mutated the Glu-63 residue to Gln (E63Q), which would be expected to block the formation of the first tetrahedral intermediate and other essential catalytic functions of this residue (Figures 1 and 5). These mutations abolished editing activity totally. Wild type and H61R, H61S, and E63Q mutants retained some ability to bind zinc, whereas none of the mutants C93A, C96A, C93A and C96A combined, and deletion of Cys-93 to Cys-96 bound zinc. This is consistent with mutations of comparable residues of the E. coli cytidine deaminase in which C129A and C132A failed to bind zinc but H102Q and H102N continued to bind zinc (Smith et al., 1994), presumably through the paired cysteine at the active site. These results indicate that the zinc-binding and catalytic mechanism of p27 is similar to that of the E. coli cytidine deaminase. p27 is therefore established as a member of the family of enzymes that act on monomeric nucleotide and nucleoside substrates.

p27, along with other factors, has acquired the ability to deaminate *apoB* mRNA in a site-specific manner, but p27 no longer has cytidine deaminase activity against monomeric substrate except when expressed in Xenopus laevis oocytes (unpublished data; Navaratnam et al., 1993a). We show here that p27 binds RNA, whereas the E. coli enzyme does not. p27 needs the zinc-binding residues His-61 and Cys-93 and the proton-shuttling residue Glu-63 (Figures 1 and 6) to bind *apoB* mRNA, but does not require Cys-96. In contrast, Cys-96 is required for zinc coordination and for RNA editing, demonstrating dissociation of the catalytic and RNA-binding function of the active site residues of p27. RNA binding by p27 does not require a functional active site. RNA editing, however, requires a functional active site that binds both zinc and *apoB* mRNA.

Phe-66 and Phe-87 are important for RNA cross-linking to p27 and for RNA editing. These residues reside in the αβα segment that encompasses the active site of the enzyme and are conserved between the rat and human catalytic subunits, and in no other deaminases. The phenylalanines at positions 70 and 77 in the rat and human enzymes do not appear to be involved in RNA–p27 interaction. In the recently reported sequence of the rabbit editing enzyme, Phe-66 and Phe-87 are conserved, but Phe-70 and Phe-77 are not (Yamanaka et al., 1994). Thus, p27 binds *apoB* mRNA through residues both in and around its active site, and RNA binding by p27 is a prerequisite for editing.

Although the structure of p27 is unlike that of other RNA-binding proteins (Burd and Dreyfuss, 1994), aromatic residues have been implicated in the UV cross-linking of RNPs (RRMs) to the small nuclear RNAs involved in splicing and other RNA-processing events. Histidine has been similarly (though less frequently) implicated. These aromatic groups probably stack with the nucleotide bases of the RNA. Proteins such as TFIIIA that use zinc to stabilize protein structure, rather than in catalysis, bind RNA through zinc fingers. However, it seems unlikely that zinc per se is involved in *apoB* mRNA binding, since both C93A and C96A fail to bind zinc, but C93A abolishes UV cross-linking while C96A does not. Also, H61S abolishes UV cross-linking, but not zinc binding. The importance of the proton-shuttling residue Glu-63 in RNA binding is unexpected, since RNA is highly negatively charged. It is possible that Glu-63 is located in proximity to other residues that it helps to position for RNA binding, by association with phosphate oxygens from the substrate RNA, and that the negative charge of this residue is neutralized. On the other hand, Glu-63 could bind directly to the bases in the RNA, as has recently been shown for MyoD binding to target DNA, in which Glu-118 makes a water and hydrogen bond–mediated contact to the base residues (Ma et al., 1994). Other parts of p27 may also be involved in RNA binding. Deletion of 14 amino acids from the amino terminus and 5 amino acids from the carboxyl terminus abolish RNA editing and UV cross-linking (unpublished data), suggesting that these regions may also contribute to the interaction of p27 with the RNA.

A number of enzymes interact with RNA. These include the iron regulatory protein/aconitase, thymidylate synthase, dihydrofolate reductase, catalase, glyceraldehyde-3-phosphate dehydrogenase, glutamate dehydrogenase, and lactate dehydrogenase (Hentze, 1994). Although the amino acids that are directly involved in RNA binding are not known for any of these enzymes, Hentze (1994) identified a striking common denominator in that their catalytic reactions involve mono- or dinucleotides as substrates or cofactors, or that their structure contains occult nucleotide-binding sites such as are found in aconitase and catalase. A feature of the first three of these enzymes is that nucleotides compete for RNA binding, suggesting that the nucleotide-binding sites and RNA-binding sites may overlap. It has been suggested that enzymatic domains that interact with nucleotides could have evolved into binding surfaces for polyribonucleotides. Indeed, RNA-binding domains with homology to (di)nucleotide-binding sites do not appear necessarily to still bind the dinucleotide itself as with aconitase and catalase. This is probably also the case with p27. The E. coli p27 fusion protein and baculovirus-expressed form no longer have cytidine deaminase activity. Also, cytidine and its analogs do not affect RNA editing (unpublished data; Driscoll et al., 1989; Greeve et al., 1991). Thus, as Hentze has suggested, the relationship between (di)nucleotide binding and RNA binding provides an evolutionary pathway for the development of RNA binding from a more simple monomeric or dimeric nucleotide binding function. It appears that this editing enzyme has

evolved because of mutations that have facilitated binding to RNA and that active site residues involved in cytidine nucleoside/nucleotide binding and deamination are also involved in RNA binding and deamination. However, a functional active site is not required for RNA–protein interaction. Additional aromatic residues are needed for RNA–protein interaction and editing, but mutation of the phenylanaline residues involved in RNA binding is not sufficient to restore cytidine deaminase activity against monomeric substrates.

Previously, we have studied the nucleotides necessary for the editing of *apoB* mRNA at C6666 in vitro. A sequence of 26 nucleotides that is highly conserved between species is required for editing (positions 6662–6687) (Davies et al., 1989). This sequence contains a core element of 11 nucleotides (positions 6671–6681), placed 5–15 nt downstream of the edited C6666, which if mutated abolishes editing (Backus and Smith, 1991; Shah et al., 1991). Upstream and downstream of this core element, mutation of the adjacent hexanucleotide segments substantially reduces editing. We now show that p27 associates with an AU-rich sequence (positions 6678–6683) overlapping with and downstream of this core sequence and less strongly with an AU-rich sequence upstream (positions 6656–6661) of the editing site. The presence of the upstream AU sequence is not needed for editing, whereas the downstream sequence is needed for editing (Davies et al., 1989; Backus and Smith, 1991; Shah et al., 1991). p27 binding to the downstream AU-rich sequence appears to be of relatively low specificity, as mutagenesis to its complement reduces but does not abolish editing (Shah et al., 1991). This view is supported by the competition studies with poly(A,U) and poly(U) RNA reported here. The high AU content of the overall sequence surrounding the editing site may serve to guide p27 to this region of the RNA (Davies et al., 1989), but the upstream AU sequence may also compete with the downstream sequence as mutagenesis of the upstream sequence to reduce its AU content enhances editing in vitro (Backus and Smith, 1991; Shah et al., 1991).

The findings that p27 binds through its active site to an AU sequence downstream of the edited C suggests that this sequence and the edited C must be brought into close proximation in the substrate RNA. A role for the active site in AU-rich sequence binding might prevent this region of the substrate from interfering with the primary RNA-binding site at C6666. p27 may dimerize or multimerize with itself, as does the E. coli enzyme, and different molecules of p27 interact with the AU sequence and edited C (Maley et al., 1990; Lau et al., 1994).

p27 alone is not competent for editing. It requires other factors that can be derived both from tissues that express *apoB* mRNA and also from HeLa cells and mammalian liver cells that do not (Teng et al., 1993; Giannoni et al., 1994; Hughes et al., 1994; this study). This might imply that the complementing factors necessary for *apoB* mRNA editing are widely expressed because of the editing of other mRNAs, or that other nonspecific cellular components complete this activity. In either case, it is likely that recognition of the *apoB* mRNA–editing site is highly specific; otherwise, this activity would be deleterious for the genome. We have previously identified a 60 kDa protein that binds specifically to the *apoB* mRNA substrate at nucleotides 6671–6674 (UGAU) that are required for editing (Navaratnam et al., 1993b). Mutation of these nucleotides completely abolishes the binding of p60. We have speculated that p60 might be part of the RNA targeting mechanism of the editing enzyme, but in the absence of purified or expressed p60 we are not able to confirm this. It is possible that p60, in association with other proteins with chaperone or matchmaker functions, might bring the edited cytidine and the binding site of p27 into close proximation (Burd and Dreyfuss, 1994).

In conclusion, in this study, we have shown that the catalytic subunit of the *apoB* mRNA–editing enzyme is a zinc-containing cytidine residue deaminase operating by the same mechanism as the E. coli enzyme that acts on mononucleotides/nucleosides. The editing enzyme has evolved away from the enzymes that act on monomeric substrates by binding to the edited nucleotide in an oligoribonucleotide context through its active site and other residues. For editing to take place, the 27 kDa catalytic subunit of the *apoB* mRNA–editing enzyme also needs complementing nuclear factors that are expressed in cells that do not normally express or edit *apoB* mRNA. Together, these studies illustrate how structural changes in the family of enzymes acting on cytidine nucleosides/nucleotides have contributed to the origins of this type of editing through the adaptation of enzymatic function. We speculate that C to U editing in plants could have evolved by similar processes.

Experimental Procedures

Chemicals

All reagents were from Sigma unless otherwise stated.

Oligonucleotides

The following oligonucleotides were used in this study and are shown 5′ to 3′, with restriction sites or mutated codons in bold. P275Bam, GAATTCGGATCCTCCGAGACAGGCCCTGTAG; P273Bam, GGCCA**GGATCC**TCATTTCAACCCTGTGGC; P273Sal, GGCCA**GTCGAC**TCATTTCAACCCTGTGGC; 5END, ACAC**AGATCT**CAAGATGAGTTCCGAGACAG; 3TAG, CATA**GGATCC**TCATTAAGCATAATCTGGAACATCATATGGATATTTCAACCCTGTGGCCCACAG; Sal1Toggle, GGTTTCTTA**GTCGAC**AGGTGGCAC; GST1, GCATGGCCTTTGCAGGGCTG; H61S, AACACCAACAAA**TCC**GTTGAAGTCAA; H61R, ACCAACAAA**CGC**GTTGAAGTC; H61R(R), GACTTCAAC**GCG**TTTGTTGGT; E63Q, CAAACACGTT**CAA**GTCAATTTC; V64L, CACGTTGAA**CTC**AATTTCATA; V64L(R), TATGAAATT**GAG**TTCAACGTG; C93A, CTGTCCTGGAGTCCC**GCT**GGGGAGTGCTCCAG; C93A(R), CTGGAGCACTCCCC**AGC**GGGACTCCAGGACAG; C96A, AGTCCCTGTGGGGAG**GCC**TCCAGGGCCATTACAG; C96A(R), CTGTAATGGCCCTGGA**GGC**CTCCCCACAGGGACT; Δ9396, TTGTTCCTGTCCTGGAGTCCCTCCAGGGCCATTACAGAATTTTTG; Δ9396(R), CCTGGAGGGACTCCAGGACAGGAACCAG; C9396A, CTGTCCTGGAGTCCC**GCT**GGGGAG**GCC**; C9396A(R), TGTAATGGCCCTGGA**GGC**CTCCCC**AGC**GGGACT; F66L, GAAGTCAAT**TTG**ATAGAAAAATTCC; F66L(R), ATTTTTCTAT**CAA**ATTGACTTC; F70L, TAGAAAAA**TTG**ACTACAGAAAG; F70L(R), CTTTCTGTAGT**CAA**TTTTTCTA; F76L, GAAAGATAC**TTG**TGTCCAAAC; F76L(R), GTTTGGACA**CAA**GTATCTTTC; F87L, CATTACCTGG**TTG**CTGTCCTGGAG; F87L(R), CTCCAGGACAG**CAA**CCAGGTAATG.

Isolation of Human p27 cDNA

A human small intestinal library (Clontech Lab) was screened with a rat cDNA probe, using standard protocols.

Conversion Assays and Primer Extension Analysis

Conversion and primer extension assays were performed as described previously (Navaratnam et al., 1993a, 1993b), using a 208-base (nucleotides 6510–6717) rat *apoB* mRNA substrate and recombinant p27 (typically 1 μg) supplemented with 0.3 μg of complementing Mono Q fraction protein.

Partial Purification of p27 Complementing Activity

Editing extracts were prepared as previously described from rat intestinal epithelium (Navaratnam et al., 1993a, 1993b) with the following modifications. Active fractions from the heparin–Sepharose were pooled and applied to poly(U)–Sepharose preequilibrated in buffer A (20 mM HEPES, 1 mM EDTA, 1 mM β-mercaptoethanol [pH 7.9]). The column was washed in buffer A, followed by buffer A with 1 mg/ml heparin (Sigma), followed by three column volumes of buffer A. The column was eluted by a 0.2 to 0.6 M linear NaCl gradient in buffer A. Fractions containing p27 complementing activity were pooled, dialyzed against buffer A, and applied to Mono Q Sepharose (Pharmacia) preequilibrated in buffer A. This was eluted with 0 to 0.6 M linear NaCl gradient. The fractions eluted from the Mono Q lack intrinsic editing activity, but can complement the p27 catalytic subunit.

HeLa Cell Nuclear Extract

HeLa cell nuclear extract was purchased from Promega and was dialyzed against 20 mM HEPES buffer (pH 7.9) containing 1 mM EDTA, 10% glycerol and was used directly in the experiments.

Expression of p27 in E. coli

p27 was expressed in E. coli as a GST fusion protein. The p27 insert was produced by polymerase chain reaction (PCR) from the cDNA sequence as described (Teng et al., 1993), using oligonucleotides P275Bam and P273Bam using Pfu polymerase (Stratagene). The PCR product was digested with BamHI, ligated into pGEX2T/BamHI (Pharmacia), and transformed into E. coli DH5αF′ cells, and the DNA sequence of the insert was verified. Cells were grown to an OD of 0.7 and induced with 0.05 mM IPTG for 30 min at 28°C. GST–p27 was affinity purified using glutathione–Sepharose (Pharmacia) following the instructions of the manufacturer. Protein content was assayed using the Bradford reagent (Bio-Rad).

Expression of p27 in Sf9 cells

A p27 insert with a 3′ HA tag was produced by PCR as above, using oligonucleotides 5END and 3TAG, and cloned into pVL1393/BamHI that was cotransfected into Sf9 cells using the Baculogold kit (Pharmingen). Recombinant virus was transfected as recommended by the manufacturer, cells were harvested after 3 days, lysed in buffer A with Triton 0.1%, and centrifuged, and the supernatant collected.

Site-Directed Mutagenesis

Mutants H61S and E63Q were made in GST–p27 using the unique site elimination technique (Deng and Nickloff, 1992) with the oligonucleotides (H61S and H61R) and the SalI–Toggle, which converts a unique AatII site in the pGEX2T/p27 construct to SalI. The other mutants were made by PCR using the splicing by overlap extension technique. Complementing mutagenic oligonucleotides (e.g. V64L and V64I(R)) were used to generate two overlapping PCR products, using the upstream primer GST1 and the downstream primer p273SalI. The overlapping products were isolated by agarose gel electrophoresis and spliced in a further round of PCR. The product was digested with BamHI and SalI, and directionally cloned into pGEX4T3/BamHI/SalI. The amino acid sequence of the GST–p27 junction is thus identical in all constructs. All mutant constructs were sequenced to confirm the mutated residue and the absence of adventitious mutations. Fusion proteins were expressed as above.

Chelation and Zinc Binding Studies

Stock solutions (1 M) of 1,10- and 1,7-o-phenanthroline (Sigma) were prepared in 100% ethanol. To 594 μl of Mono Q fraction or Sf9 supernatant, 6 μl of 1,10- or 1,7-o-phenanthroline or ethanol was added. The mixture was incubated at 30°C for 10 min, and 50 μl aliquots were removed and stored at 4°C. The remainder was dialyzed against buffer A containing 10 mM β-mercaptoethanol overnight. Aliquots (50 μl) were removed, and dialysis was continued in the same buffer containing 5 mM zinc sulphate for 6 hr, following which the buffer was changed to buffer A and dialysis continued overnight. Aliquots were then assayed for editing activity.

Zinc Binding Studies

Wild-type and mutant GST fusion proteins were purified using glutathione–Sepharose. The matrix with bound proteins was washed by rocking overnight with 100 bed volumes of phosphate-buffered saline at 4°C. Purified fusion proteins were cleaved with thrombin in the presence of 20 μM heparin overnight at 25°C. The supernatant was concentrated on a Centricon C-10 (Armicon), fractionated on a 12% gel by SDS–PAGE at 4°C, and blotted onto nitrocellulose membrane in CAPS (pH 11), methanol 10% buffer for 75 min. Proteins on the blot were denatured overnight in metal binding buffer (Mazen et al., 1988) containing 6 M guanidium hydrochloride and 1% β-mercaptoethanol at 4°C and renatured in six steps by diluting the guanidium with an equal volume of metal binding buffer containing 1% β-mercaptoethanol, followed by two washes with metal binding buffer containing 1% β-mercaptoethanol. The denaturation/renaturation step markedly improved the zinc binding signal. Following the final step, the blot was rinsed twice with metal binding buffer and then incubated with $^{65}ZnCl_2$ (Amersham) at 1 μCi/ml in metal binding buffer for 30 min at room temperature. The blot was washed in eight changes of metal binding buffer for 30 min, air dried, and autoradiographed on Kodak X-OMAT using a screen at −70°C for 8 hr.

UV Cross-Linking Studies

Wild-type (A–I) and deletion (A–C, A–D, A–E, C–E, E–G, E–H, E–I, F–I, 5′ΔDF, 3′ΔDF, and G–I) RNA transcripts used in the UV cross-linking studies were synthesized by T7-directed in vitro transcription reactions in the presence of [α-^{32}P]UTP (Amersham). All reagents used were obtained from Ambion Biotechnology Limited. Templates for T7 transcription were generated by PCR (Milligan et al., 1987). HindIII-linearized plasmid pBS 55 (Shah et al., 1991) was used as the template in the PCR reactions. 5′ oligonucleotides for PCR were generated by appending a 23 nt T7 promoter sequence (GGA TCC TAA TAC GAC TCA CTA TAG GGA TCC GG) to 5′ end (12 nt) of the desired template. 3′ oligonucleotides were complementary to the required 3′ end of the template. UV cross-linking studies were performed as before (Navaratnam et al., 1993b). GST–p27 wild-type or mutant protein (~1 μg) was incubated with ^{32}P-labeled RNA at 30°C for 20 min and UV cross-linked for 10 min on ice. Cross-linked products were separated on 10% or 12% SDS–PAGE gels. Gels were stained with Coomassie blue, and dried gels were subjected to autoradiography.

Competition studies were performed as previously described (Shah et al., 1991) with polyribonucleic acids.

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