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Regulation of escherichia coli ribonuclease III by the bacteriophage T7 protein kinase

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REGULATION OF *ESCHERICHIA COLI* RIBONUCLEASE III BY THE BACTERIOPHAGE T7 PROTEIN KINASE

by

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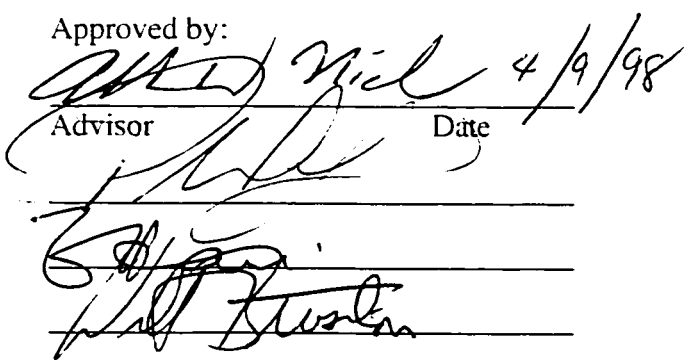

Advisor Date

DEDICATION

This dissertation is dedicated to the Birmingham Four, the Roots, Lady Bug, Lee Allen, and all the unborn seeds.

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ABBREVIATIONS

Adh	Alcohol dehydrogenase
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
bp	Base pair
BSA	Bovine Serum Albumin
cAMP	Adenosine 3',5'-cyclic monophosphate
cGMP	Guanosine 3',5'-cyclic monophosphate
cpm	Counts per minute
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dsDNA	Double-stranded-Deoxyribonucleic Acid
dsRBD	Double-stranded-Ribonucleic Acid-Binding Domain
dsRNA	Double-stranded Ribonucleic Acid
DTT	Dithiothreitol
EDTA-Na_2	Ethylene Diaminetetraacetic Acid, Disodium salt
EI	Enzyme I of Ntr system
EIII	Enzyme III of Ntr system
GTP	Guanosine Triphosphate
IDH	Isocitrate Dehydrogenase
IEF	Isoelectric Focusing
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria-Bertani
mA	Milliamperes
m.o.i.	Multplicity of infection
MOPS	3-[N-morpholino]propanesulfonic acid
M_r	Molecular weight (relative)
mRNA	Messenger Ribonucleic Acid
NMR	Nuclear Magnetic Resonance

NRI	Nitrogen Regulatory Protein I
Ntr	Nitrogen Regulatory System
PAGE	Polyacrylamide Gel Electrophoresis
PK	Protein Kinase
PTS	Phosphotransferase system
RNA	Ribonucleic Acid
RBS	Ribosome Binding Site
RNase	Ribonuclease
rnc	Ribonuclease III structural gene
rrf	Ribosomal Ribonucleic Acid 5S gene
rrl	Ribosomal Ribonucleic Acid 23S gene
rrn	Ribosomal Ribonucleic Acid operon
rRNA	Ribosomal Ribonucleic Acid
rrs	Ribosomal RNA 16S gene
SDS	Sodium Dodecylsulfate
ssRNA	Single stranded Ribonucleic Acid
TBE	Tris-Borate- Ethylene Diaminetetraacetic Acid, Disodium salt
TCA	Tricarboxylic acid cycle
tRNA	Transfer Ribonucleic Acid
2-D	2-Dimensional
UTP	Uridine Triphosphate
UTR	Untranslated Region
UV	Ultraviolet
V	Volts
W-C	Watson-Crick
wt	Wild type

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INTRODUCTION

The covalent modification of protein controls a myriad of physiological processes. Protein modification reactions include: acetylation, ADP-ribosylation, methylation, nucleotidylation, sulfation, tyrosylation, and phosphorylation. Phosphorylation is the most common type of covalent modification (Hanks *et al.*, 1988; Hershey, 1989; Hershey, 1991; Hanks and Quinn, 1991). The number of proteins in the eukaryotic cell which are regulated by phosphorylation is enormous. In comparison, the number of bacterial proteins which are regulated by phosphorylation is small (Deutscher and Saier, 1988). However, this number is quickly increasing. Well characterized bacterial proteins that are regulated by phosphorylation include: isocitrate dehydrogenase, HPr, glycerol kinase, nitrogen regulatory protein I, and citrate lyase (Table 1) (Deutscher and Engelman, 1984; Cortay *et al.*, 1988; Deutscher and Saier, 1988; Deutscher and Sauerwald, 1986; Ninfa and Magasanik, 1986; Saier *et al.*, 1990).

The involvement of protein phosphorylation in regulating physiological processes in the eukaryotic cell was first demonstrated during the mid-1950s, where it was shown that glycogen phosphorylase, which converts glycogen to glucose-1-phosphate molecules, exists in two forms: phosphorylated and unphosphorylated (Krebs and Fisher, 1956). Phosphorylase kinase phosphorylates glycogen phosphorylase, thereby activating the enzyme. Active glycogen phosphorylase is then able to catalyze the phosphate-dependent release of glucose-1-phosphate from glycogen. Since that time, many protein kinases, phosphoproteins and protein phosphatases have been discovered. Several early investigations attempted to determine whether protein phosphorylation occurred in the prokaryotic cell. The results were negative, and for several decades it was generally accepted that protein phosphorylation occurred exclusively within the eukaryotic cell (Li and Brown, 1973; Pastan and Adhya, 1976). It was theorized that phosphorylation-dephosphorylation regulatory mechanisms were an evolutionarily recent development.

Table 1. Prokaryotic Cellular Reactions Controlled by Protein Phosphorylation

Cellular Process	Kinase	Gene	Mass (kDa)	Target of Kinase	Phosphoamino acid	Result of Phosphorylation
Ntr System	NRI kinase	*glnG*	110	NRI	Serine	Transcription of *glnA* gene
TCA cycle	IDH kinase	*aceK*	66	IDH	Serine	Activation of glyoxylate bypass
PTS	EI	*ptsI*	58	HPr	Histidine (N-1)	Sugar uptake
PTS	P-HPr	*ptsH*	9	EIII	Histidine (N-3)	Sugar uptake
Glycerol uptake	P-HPr	*ptsH*	9	Glycerol Kinase	Histidine (N-3)	Phosphorylation of glycerol
β–glucoside utilization	BglF	*bglF*	29	BglG	Histidine	BglG protein inactivation

designed to meet the needs of multicellular organisms having differentiated cells with compartmentalized subcellular structures (Cozzone, 1984). Within the last two decades this conclusion has proven to be incorrect. Over fifty different species of bacteria exhibit reactions involving protein phosphorylation, and more than a hundred phosphoproteins have been detected in the gram negative bacterium, *Escherichia coli* (Cortay *et al.*, 1986). It is now accepted that protein phosphorylation is a universal cellular process. A current theory is that protein phosphorylation arose very early in cellular evolution. This theory derives from sequence alignment analysis, which indicate that protein kinases in prokaryotes and eukaryotes descended from a single prototype (Kennelly and Potts, 1996).

Most protein kinases are ATP-dependent, in that they catalyze the transfer of the γ phosphate of ATP to an acceptor substrate (Scheme 1). However, some protein kinases are GTP-dependent, while still others utilize phosphoproteins as phosphate donors (Deutscher and Saier, 1983). Still other protein kinases utilize small metabolites such as phosphoenol pyruvate, acetyl phosphate, or carbamoyl phosphate as phosphate donors (Saier *et al.*, 1990; Lukat *et al.*, 1992). Many eukaryotic protein kinases are regulated by secondary messengers (cyclic AMP, cyclic GMP, Ca^{2+}/calmodulin, $NADH^{+}$). Secondary messengers also appear to be involved (although to a lesser extent) in the regulation of prokaryotic protein kinases (Edelman, 1987; Taylor, 1990).

Scheme 1. Protein Phosphorylation/Dephosphorylation.

(i) phosphorylation by a protein kinase (PK)

(ii) dephosphorylation by a protein phosphatase (PP)

(i) ATP + PK + Protein $\Rightarrow$ ADP + PK + Protein-PO_3^{2-}

(ii) H_2O + Protein-PO_3^{2-} + PP $\Rightarrow$ Protein + PP + HPO_4^{2-}

Protein kinases can be classified into three main groups, based on acceptor amino acid specificity. Thus, there are serine/threonine-specific protein kinases, histidine-specific

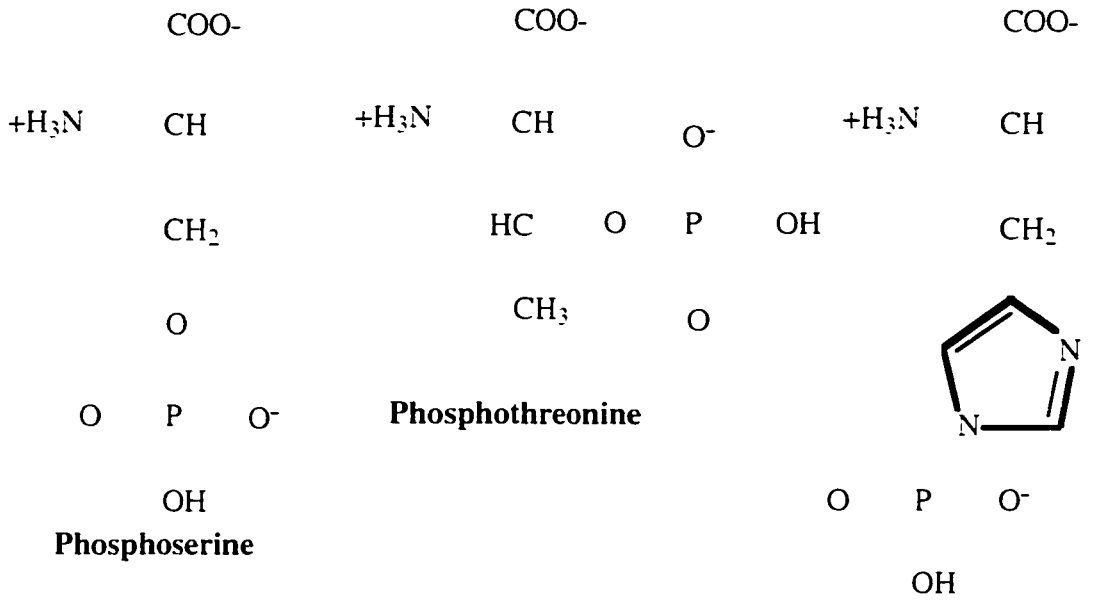
protein kinases, and tyrosine-specific protein kinases (minor protein kinase groups include those specific for lysine or aspartic acid residues) (Figure 1). Phosphotransfer to serine, threonine, or tyrosine side chains create phosphoester bonds which are chemically stable at physiological pH (~7). However, protein kinases do not phosphorylate any accessible serine/threonine, tyrosine, or histidine residue. The particular amino acid side chain target is identified by specific sequence elements (usually next to the target site) in addition to accessibility of the acceptor side chain (Kemp and Pearson, 1990).

Based on sequence comparisons, eukaryotic protein kinases are grouped in a single enzyme "superfamily", which exhibits a core 250 amino acid "catalytic domain" (Hanks and Quinn, 1991). Within this superfamily, the serine/threonine protein kinases represent the major subgroup, with phosphorylation occurring on serine more frequently than on threonine. Prokaryotic protein kinases have been discovered which share sequence similarity with the eukaryotic protein kinase catalytic domain. These include protein kinase Pkn1 of *Myxococcus xanthus* (Muñoz-Dorado *et al.*, 1991), and protein kinases PkaA, PkaB, and AfsK of *Streptomyces coelicolor* (Urabe and Ogawara, 1995).

Protein kinases introduce a negative charge in the target protein. The negative charge probably is the most significant functional consequence of phosphorylation. For example, the phosphate group is capable of attracting positively charged protein side chains, causing a protein conformational change (Schultz, 1990). Alternatively, introduction of a negative charge can cause electrostatic repulsion with other acidic (i.e., negatively charged) protein side chains. The charge-induced conformational change can alter target protein activity.

A. Role of Protein Phosphorylation in the Bacterial Cell.

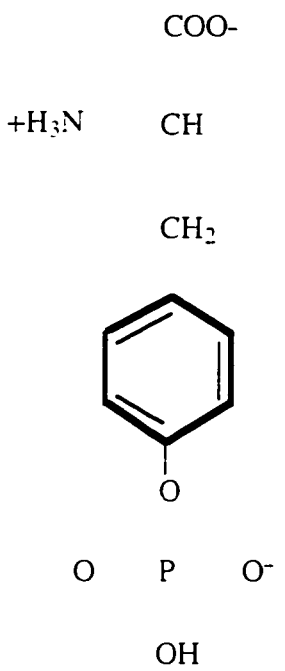
Bacterial cells exhibit rapid adaptive responses. Noxious agents, or the availability of carbon, nitrogen, phosphate, amino acids, or vitamins can elicit a specific response (for example: chemotaxis, nitrogen uptake regulation, phosphate uptake regulation, osmoregulation, sporulation). Histidine protein kinases are often involved in these



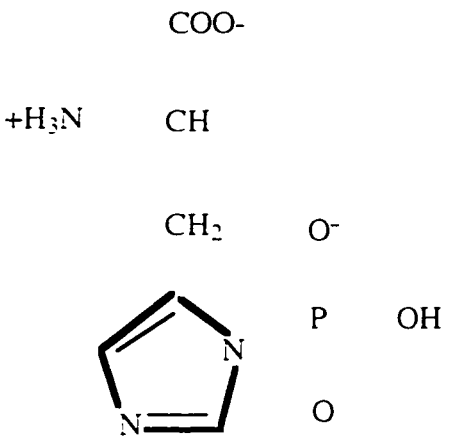
Phosphoserine

Phosphothreonine

Phosphohistidine (N-3)



Phosphotyrosine



Phosphohistidine (N-1)

Figure 1. Phosphorylation of protein serine, threonine, histidine, and tyrosine side chains.

responses, and can also control gene expression, cell motility, and cell morphology (Stock *et al.*, 1989). Basic steps in adaptive responses occur in the following general order: 1) an environmental stimulus causes a sensory protein kinase to autophosphorylate; 2) the activated sensory protein kinase transfers the ATP γ phosphate to the N1 or N3 atom of histidine (Figure 1) on a histidine protein kinase (HPK-his); 3) the histidine protein kinase phosphorylates an aspartic acid side chain of a response regulator protein (RR-asp); and 4), the phosphate on the phosphoaspartic acid side chain of the response regulator protein is transferred to water (Scheme 2). Adaptive response mechanisms can involve several protein kinases, several regulator response proteins, or both. In other adaptive responses, a single protein acts as the histidine kinase as well as the regulator response protein (McLeary and Zusman, 1990).

Scheme 2. Protein phosphorylation in bacterial adaptive responses.

(i) Autophosphorylation on histidine of a histidine protein kinase (HPK-his)

(ii) Phosphorylation on aspartic acid of regulator response protein (RR-asp) and

(iii) Dephosphorylation of regulator response protein by protein phosphatase (PP)

(i) $\mathbf{ATP + HPK\text{-}his \Rightarrow ADP + HPK\text{-}his\text{-}PO_3^{2-}}$

(ii) $\mathbf{HPK\text{-}his\text{-}PO_3^{2-} + RR\text{-}asp \Rightarrow HPK\text{-}his + RR\text{-}asp\text{-}PO_3^{2-}}$

(iii) $\mathbf{H_2O + RR\text{-}asp\text{-}PO_3^{2-} \Rightarrow RR\text{-}asp + HPO_4^{3-}}$

There are only a few examples of tyrosine phosphorylation in bacteria. With the discovery of phosphotyrosine in acid hydrolysates of bacterial cell extracts (Manai and Cozzone, 1983; Cortay, 1986; Vallejos *et al.*, 1985; Londesborough, 1986), it was proposed that these residues might be derived from nucleotidylated tyrosines (Foster *et al.*, 1989). Tyrosine phosphorylation has subsequently been confirmed directly in several

bacterial systems through the use of antiphosphotyrosine antibodies and differential radiolabeling (Warner and Bullerjahn, 1994; Walters *et al.*, 1994).

As discussed above, protein kinases control diverse cellular reactions. There is now growing evidence for the control of cellular and viral RNA metabolism by protein phosphorylation.

B. Biological Roles of RNA.

The cell expends a considerable amount of energy and resources synthesizing, modifying, regulating, and degrading RNA. For example, ~20% of the *Escherichia coli* cell dry mass is RNA (Neidhardt *et al.*, 1990). When the "Central Dogma" of molecular biology was proposed in the mid-1950s (Crick, 1958), it was assumed that all RNA was template RNA (that is, the primary function of RNA was to encode polypeptide sequences). The subsequent discovery of transfer RNA and ribosomal RNA filled in major gaps of knowledge of the mechanism of gene expression. By the late 1970s, the structures and biological functions of the three major functional classes of cellular RNA (transfer, ribosomal, and messenger RNA) were established. The first major modification of the central dogma occurred with the discovery of reverse transcription, where genetic information in the retroviruses was shown to flow from RNA to DNA (Baltimore, 1970; Temin and Mizutani, 1970). A second modification stemmed from the discovery of introns in eukaryotic genes, and RNA splicing (Breathnach *et al.*, 1977; Jeffreys and Flavell, 1977). RNA splicing forced a modification of the concept of the colinearity of gene and protein sequence. Introns were also discovered in tRNAs as well as rRNAs (Abelson, 1979; Gegenheimer and Apirion, 1981; Nakajima *et al.*, 1981). A third major development in the field of RNA biology was the discovery of catalytic RNA (the RNA subunit of *E. coli* RNase P, and the *Tetrahymena* self-splicing rRNA intron) (Kruger *et al.*, 1982; Guerrier-Takada *et al.*, 1983; Cech, 1993).

C. RNA Processing and Ribonuclease III.

Many RNA molecules must undergo one or more processing steps to achieve their mature, functional forms. In addition, all RNA molecules are ultimately degraded to mononucleotides. These reactions are carried out by cellular ribonucleases, which include endoribonucleases and 3'→5' exoribonucleases (Nicholson, 1997) (Table 2).

RNase III is a double-stranded-RNA-specific endoribonuclease, first detected in *E. coli* cell-free extracts (Robertson *et al.*, 1968). RNase III is involved in the maturation of the 16S and 23S rRNAs (Srivastava *et al.*, 1992), the maturation and degradation of cellular mRNAs (Dunn, 1982; Court, 1993), and the maturation and degradation of viral mRNAs (Dunn and Studier, 1973a,b; Dunn, 1982; Court, 1993).

RNase III functions as a homodimer of a 25.6 kDa polypeptide which is 226 amino acids long (Figure 2). The RNase III polypeptide consists of two functional domains; a ~148 residue "catalytic" domain, and a ~78 residue, carboxyl-terminal proximal, double-stranded-RNA-binding-domain (dsRBD). These two domains have been separately overexpressed and purified, and exhibit RNA cleavage and binding activities, respectively (A. Amarasinghe and E-J. Jun, unpublished). Biochemical assays of the RNase III dsRBD have shown that it can bind but cannot cleave dsRNA (A. Amarasinghe *et al.*, in preparation). A homonuclear NMR study by Pastore and coworkers (Kharrat *et al.*, 1995) revealed that the RNase III dsRBD has an α_1–β_1–β_2–β_3–α_2 supersecondary structure. The three β strands form an antiparallel β-sheet, with the two α helices packed against one side of the β-sheet. On one surface of the domain there are two loops: loop 2, which connects β_1 to β_2, and loop 4, which connects β_3 to α_2, which are rich in positively charged amino acids, and may participate directly in dsRNA binding (Kharrat *et al.*, 1995) (Figure 3).

The structure of the catalytic domain is not known. Biochemical experiments have shown that the isolated catalytic domain can cleave substrate at the same sites recognized by the holoenzyme (E-J. Jun, unpublished). The catalytic domain therefore contains the active

Table 2. ***Escherichia coli*** **Ribonucleases**

Ribonuclease	Gene	Mass(kDa)	Substrates	Function
RNase P	*rnpA* **rnpB*	14 M1 RNA, 377nt	pre-tRNAs	tRNA maturation
RNase III	*rnc*	25.6	dsRNA	mRNA, rRNA maturation and decay
RNase E	*rne*	120	mRNA, 9S rRNA	5S rRNA maturation, mRNA degradation
RNase I	*rna*	27	ssRNA	RNA degradation
RNase HI	*rnhA*	17.6	RNA/DNA hybrids	Replication, DNA repair
RNase HII	*rnhB*	23.2	RNA/DNA hybrids	---
RNase M	---	27	ssRNA	---
RNase R	---	24	ssRNA	---
RNase IV	---	31	ssRNA	tRNA maturation
RNase N	---	120	ssRNA, dsRNA	---
RNase II	*rnb*	70	ssRNA, poly(A)	mRNA degradation, tRNA maturation
PNPase	*pnp*	85	ssRNA	mRNA degradation, tRNA maturation
RNase D	*rnd*	42.7	pre-tRNAs	tRNA maturation
RNase BN	*rbn*	60	pre-tRNAs	tRNA maturation
RNase T	*rnt*	23.5	pre-tRNAs	tRNA maturation
RNase PH	*rph*	25.5	pre-tRNAs	tRNA maturation
Oligo RNase	---	38	oligo-ribonucleotides	RNA degradation
RNase R	---	80	rRNA	RNA degradation

site, and the dsRBD most likely allows RNase III to bind substrate more efficiently, and perhaps activate the catalytic domain.

RNase III functions as a phosphodiesterase, using a water molecule as nucleophile to create 5'-phosphate and 3'-hydroxyl RNA product termini (Dunn, 1976; 1982). RNase III requires a divalent metal ion for activity. Although Mg^{2+} is the preferred ion, Mn^{2+}, Co^{2+}, and Ni^{2+} will also support cleavage of substrate (Li *et al.*, 1993). RNase III cleavage of substrate exhibits Michaelis-Menten steady-state kinetics (Li *et al.*, 1993).

RNase III is encoded by the single copy *rnc* gene, which maps at 55 minutes on the *E. coli* chromosome. The *rnc* gene is the first gene in the *rnc* operon (Matsunaga *et al.*, 1996), which also contains the *era* and *recO* genes (Figure 6). The *era* gene encodes a GTP-binding protein, Era, whose function is essential but currently unknown (March *et al.*, 1988). The *recO* gene encodes a non-essential protein (RecO) that is involved in recombination (Kolodner *et al.*, 1985; Takiff *et al.*, 1989).

A primary role for RNase III is the maturation of the ribosomal RNAs. RNase III site-specifically cleaves dsRNA structures in the primary transcripts of the rRNA operons, creating the immediate precursors to the mature 16S and 23S rRNAs. There are seven independent rRNA transcriptional units in *E. coli* (*rrnA*, *rrnB*, *rrnC*, *rrnD*, *rrnE*, *rrnG*, *rrnH*), which exhibit a high similarity in primary sequence (Brosius, 1981). The genetic organization of the seven rRNA operons is shown in Figure 4. The general structure (from left to right) includes: two strong promoters (P_1 and P_2); 5'-Leader sequence; 16S rRNA sequence; spacer sequence; tRNA sequence; spacer sequence; 23S rRNA sequence; spacer sequence; 5S rRNA sequence; 3'-trailer sequence; and two terminators (T_1 and T_2). Operons *rrnA*, *rrnD*, and *rrnH* contain two tRNA sequences between the 16S and 23S rRNAs, and in the *rrnC*, *rrnD* and *rrnH* operons there is a tRNA sequence distal to the 5S rRNA (Ikemura and Nomura, 1977; Ikemura and Ozeki, 1977). The tandem promoters P_1 and P_2 are separated by ~100 base pairs, with the downstream P_2 promoter ~200 base

```
1    MetAsnProIleValIleAsnArgLeuGlnArgLysLeuGlyTyrThrPheAsnHis
1    ATGAACCCCATCGTAATTAATCGGCTTCAACGGAAGCTGGGCTACACTTTTAATCAT

20   GlnGluLeuLeuGlnGlnAlaLeuThrHisArgSerAlaSerSerLysHisAsnGlu
60   CAGGAACTGTTGCAGCAGGCATTAACTCATCGTAGTGCCAGCAGTAAACATAACGAG

39   ArgLeuGluPheLeuGlyAspSerIleLeuSerTyrValIleAlaAsnAlaLeuTyr
117  CGTTTAGAATTTTTAGGGGACTCTATTCTGAGCTACGTTATCGCCAATGCGCTTTAT

58   HisArgPheProArgValAspGluGlyAspMetSerArgMetArgAlaThrLeuVal
174  CACCGTTTCCCTCGTGTGGATGAAGGCGATATGAGCCGGATGCGCGCCACGCTGGTC

77   ArgGlyAsnThrLeuAlaGluLeuAlaArgGlyPheGluLeuGlyGluCysLeuArg
231  CGTGGCAATACGCTGGCGGAACTGGCGCGCGAATTTGAGTTAGGCGAGTGCTTACGT

96   LeuGlyProGlyGluLeuLysSerGlyGlyPheArgArgGlySerIleLeuAlaAsp
288  TTAGGGCCAGGTGAACTTAAAAGCGGTGGATTTCGTCGTGAGTCAATTCTCGCCGAC

115  ThrValGluAlaLeuIleGlyGlyValPheLeuAspSerAspIleGlnThrValGlu
345  ACCGTCGAAGCATTAATTGGTGGCGTATTCCTCGACAGTGATATTCAAACCGTCGAG

134  LysLeuIleLeuTrpTyrGlnThrArgLeuAspGluIleSerPro|GlyAspLysGln
402  AAATTAATCCTCTGGTATCAAACTCGTTTGGACGAAATTAGCCCA|GGCGATAAACAA

153  LysAspProLysThrArgLeuGlnGluTyrLeuGlnGlyArgHisLeuProLeuPro
459  AAAGATCCGAAAACGCGCTTGCAAGAATATTTGCAGGGTCGCCATCTGCCGCTGCCG

172  ThrTyrLeuValValGlnValArgGlyGluAlaHisAspGlnGluPheThrIleHis
516  ACTTATCTGGTAGTCCAGGTACGTGGCGAAGCGCACGATCAGGAATTTACTATCCAC

191  ValCysGlnValSerGlyLeuSerGluProValValGlyThrGlySerSerArgArg
573  GTTTGCCAGGTCAGCGGCCTGAGTGAACCGGTGGTTGGCACAGGTTCAAGCCGTCGT

210  LysAlaGluGlnAlaAlaAlaGluGlnAlaLeuLysLysLeuGluLeuGlu
630  AAGGCTGAGCAGGCTGCCGCCGAACAGGCGTTGAAAAAACTGGAGCTGGAA
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Figure 2. DNA sequence of the *Escherichia coli rnc* gene and predicted amino acid sequence of RNase III. The *rnc*105 mutation changes glycine 44 (red Gly) to aspartic acid. The *rnc* 70 mutation changes glutamic acid 117 (red Glu) to lysine. The *rnc*105 and *rnc*70 RNase III mutants are both deficient in processing activity (i.e. the 30S rRNA precursor accumulates). However, the Rnc70 RNase III mutant retains specific binding to RNase III substrates (Li and Nicholson, 1996). The underlined amino acid sequence HisAsnGluArgLeuGluPheLeuGlyAspSer, may be involved in catalytic activity. The green amino acid sequence SerGlyLeuSerGluProValValGlyThrGly SerSerArgArgLysAlaGluGlnAlaAlaAlaGluGlnAlaLeu, is the highly conserved region in the dsRBD. Serine residues are highlighted in blue. Serine residues highlighted in red represent residues which have been mutated to alanine in this study. The | symbol in the sequence (between Pro 148 and Gly 149) represents the end of the catalytic domain and beginning of the dsRBD. Point mutations relevant to this study are in red.

Table 3. Cellular and Viral RNase III Substrates

RNase III substrate	Result of cleavage
rrnA,B,C,D,E,G,H transcripts	Production of pre-rRNAs, and tRNAs
rnc-era-recO transcript	mRNA decay
adhE mRNA	Activation of translation
speF-potE transcript	Stabilization of mRNA
rpsO-pnp operon	mRNA decay
metY-nusA-infB transcript	mRNA decay
T7 polycistronic mRNA	Maturation and stabilization of mRNA
λ *N* mRNA	mRNA decay
λ *int* mRNA	mRNA decay

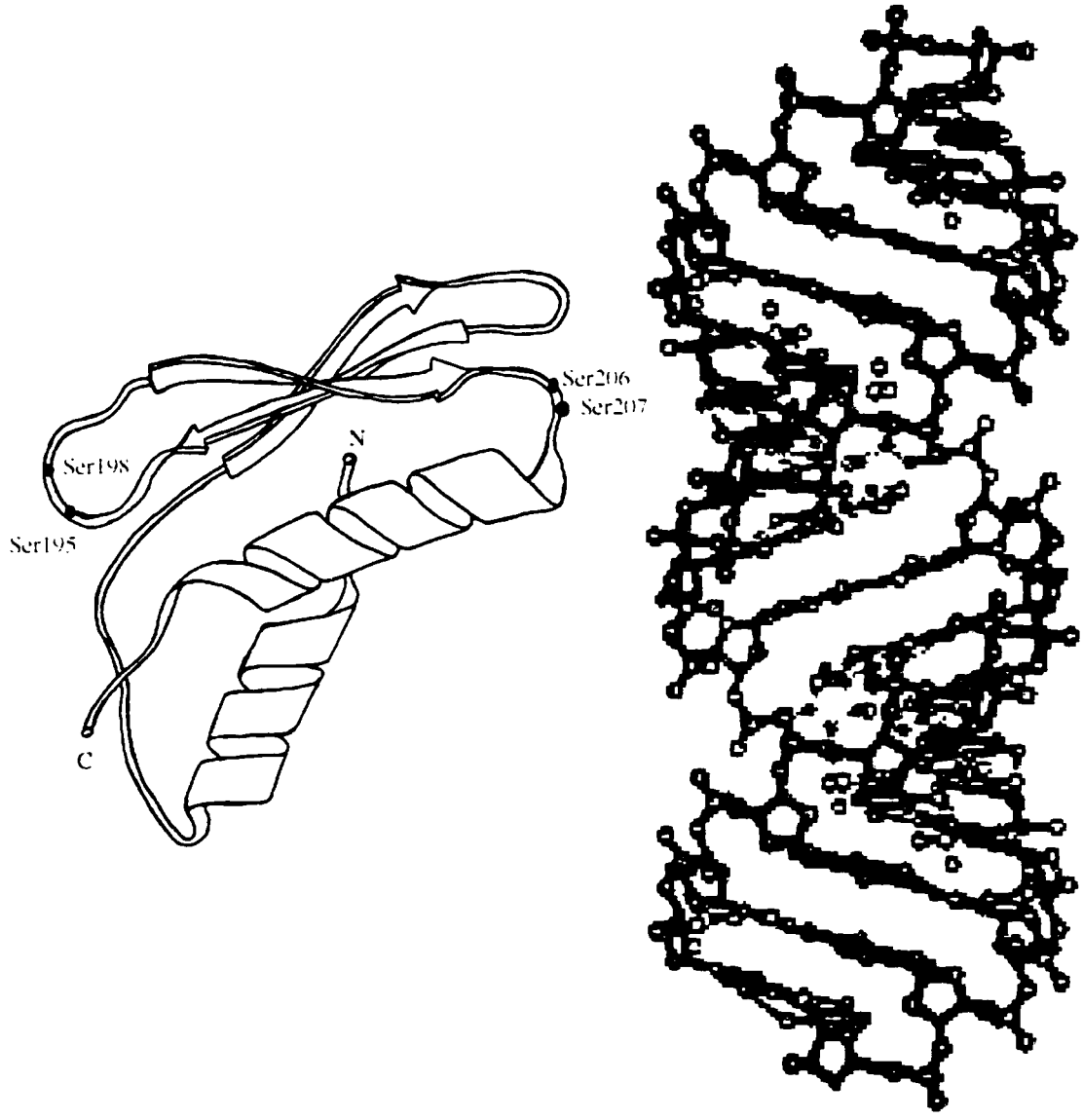
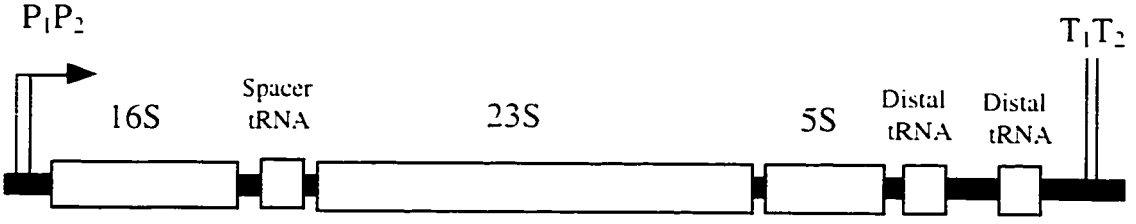


Figure 3. Structure of the dsRBD, and proposed interaction with dsRNA. Displayed is an adaptation of the dsRBD supersecondary structure as proposed by Pastore and coworkers. Homonuclear NMR studies by Pastore and coworkers (Kharrat *et al.*, 1995) revealed that the RNase III dsRBD has an α_1–β_1–β_2–β_3–α_2 supersecondary structure. Loop 2 connects β_1 to β_2 and loop 4 connects β_3 to α_2. These loops are rich in positively charged amino acids and are believed to participate in dsRNA binding (Kharrat *et al.*, 1995). The approximate positions of the four serines within the dsRBD are in red and the N- and C-terminal ends are indicated.

pairs upstream of the 16S rRNA. The P_2 promoter is responsive to control by (p)ppGpp. The P_1 promoter is the stronger of the two and is subject to growth rate control (de Boer and Nomura, 1979; Lund and Dahlberg, 1979; Glaser *et al.*, 1983; Kajitani and Ishihama, 1984). The spacer regions are highly conserved among the operons, and include long inverted repeat sequences which flank the 16S and 23S rRNAs. These inverted repeats form the double-stranded stems of the pre-16S and pre-23S rRNAs (Bram *et al.*, 1980). RNase III cleavage occurs 115 nucleotides downstream of the mature 5' end of 16S rRNA, and 33 nucleotides upstream of the mature 3' end. In the 23S rRNA, RNase III cleavage occurs 7 nucleotides downstream of the 5' end and 8 nucleotides upstream of the 3' end. These cleavages occur before transcription of the operon is complete, and provide the pre-16S and pre-23S rRNAs (Apirion, 1984). A 9S rRNA (pre-5S rRNA) is also created as a result of RNase III cleavage at the pre-23S rRNA 3' site (Figure 5). The pre-5S rRNA contains an extra 85 nucleotides at its 5' end, while the 3' end extends to the transcription terminator (Fournier and Ozeki, 1985). These pre-rRNAs are further processed by other ribonucleases, resulting in the mature rRNAs. Numerous nucleotides in the 16S, 23S, and 5S rRNAs are modified, and provide the mature rRNA species. Most of the nucleotide modifications occur in regions of high sequence conservation, indicating essential functional roles for these residues (Noller and Woese, 1981; Noller *et al.*, 1981).

E. coli mutants which lack RNase III are viable (Dunn and Studier, 1973a,b; Takiff *et al.*, 1989; Babitzke *et al.*, 1993). Ribosomes isolated from RNase III$^-$ cells contain the properly matured 16S rRNA species. However, the 5' and 3' ends of the 23S rRNA contain extra nucleotides (~20-97 nucleotides at the 5' end, and ~53 nucleotides at the 3' end) (King *et al.*, 1984). Thus, although an alternate processing pathway for 16S rRNA can function in the absence of RNase III, the 23S rRNA remains incompletely matured, albeit functional.

RNase III negatively autoregulates its expression. The primary transcript from the



E. coli map position (minutes)	Operon	16S gene	Spacer tRNA gene(s)	23S gene	5S gene	Distal tRNA gene 1	Distal tRNA gene 2
87	*rrnA*	*rrsA*	*ileT alaT*	*rrlA*	*rrfA*	---	---
90	*rrnB*	*rrsB*	*gltT*	*rrlB*	*rrfB*	---	---
85	*rrnC*	*rrsC*	*gltU*	*rrlC*	*rrfC*	*aspT*	*trpT*
72	*rrnD*	*rrsD*	*ileU alaU*	*rrlD*	*rrfD*	*thrV*	---
90	*rrnE*	*rrsE*	*gltV*	*rrlE*	*rrfE*	---	---
57	*rrnG*	*rrsG*	*gltW*	*rrlG*	*rrfG*	---	---
5	*rrnH*	*rrsH*	*ileV alaV*	*rrlH*	*rrfH*	*aspU*	---

Figure 4. Schematic diagram of the *Escherichia coli rrn* operons. The diagram indicates positions of the tandem promoters (P_1 and P_2), 16S, 23S, and 5S rRNA genes, spacer tRNA, 3' distal tRNAs, and the tandem terminators (T_1, T_2). The table provides the location and genetic elements in the seven *rrn* operons.

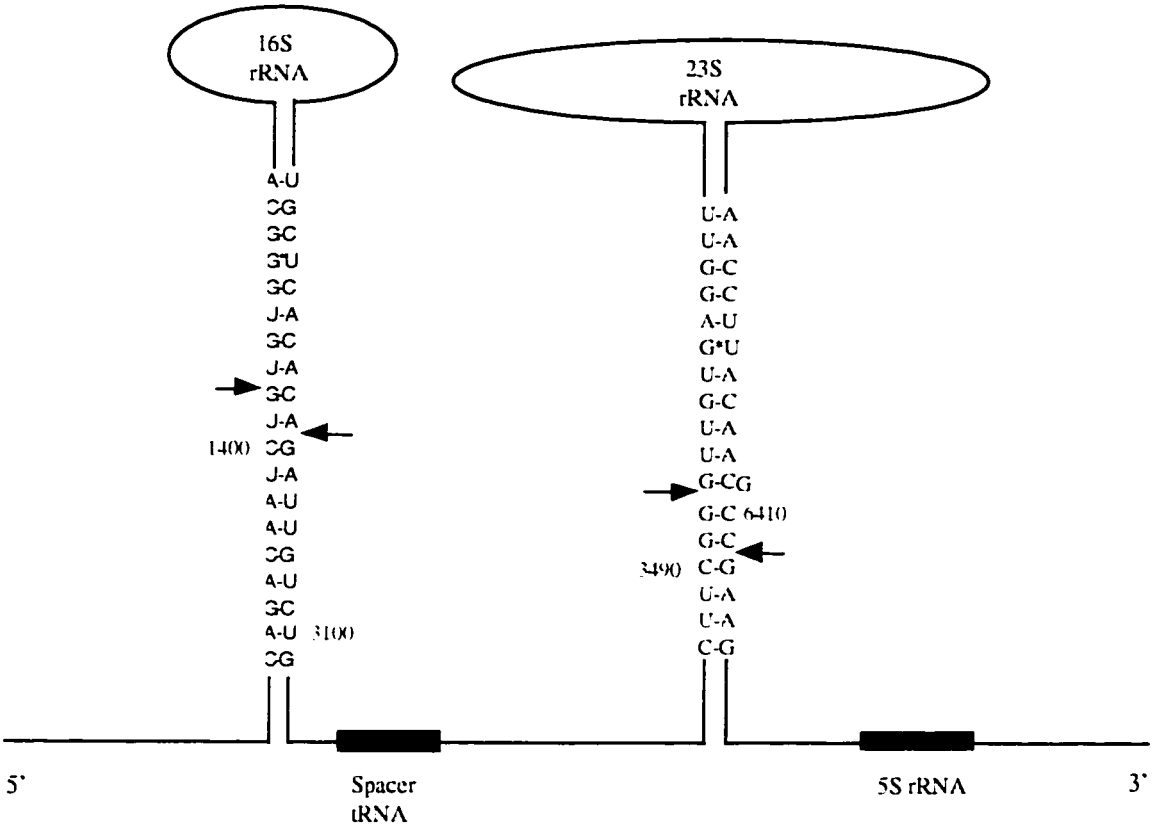


Figure 5. RNase III cleavage sites in a representative rRNA operon transcript. Blue numbers indicate nucleotide position in the operon. Red arrows indicate RNase III cleavage sites.

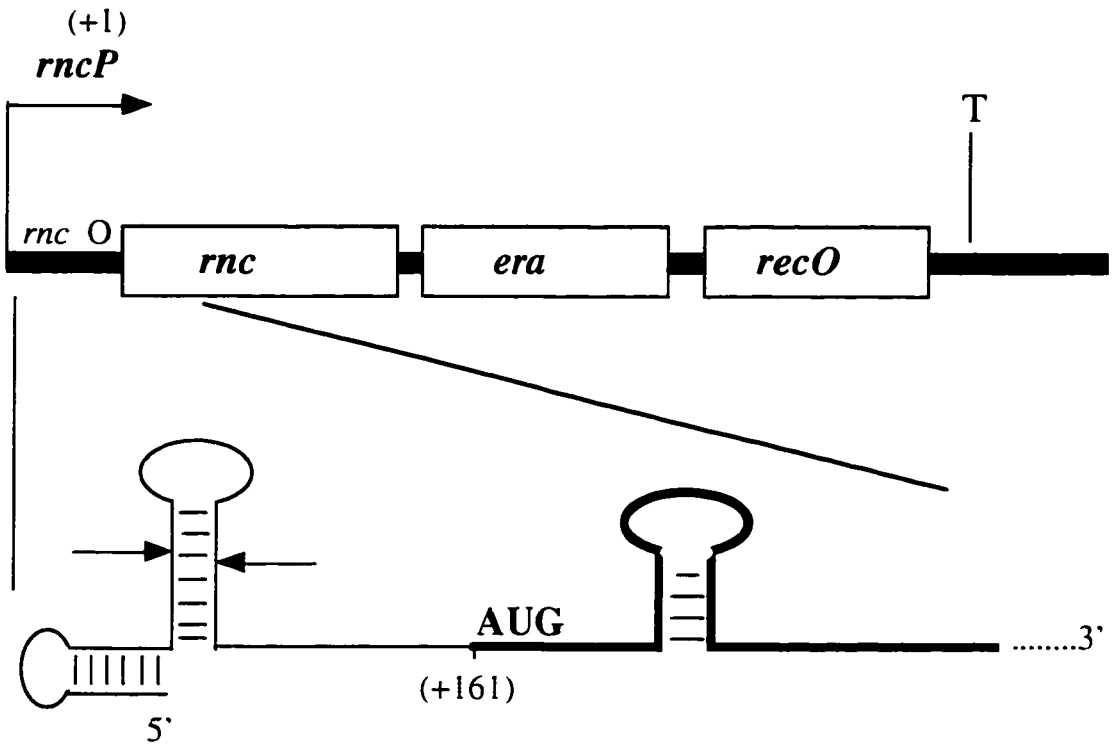


Figure 6. Schematic diagram of the *Escherichia coli rnc* operon (adapted from Matsunaga *et al.*, 1996). The diagram indicates the promoter (*rncP*), and the positions of the *rnc*, *era*, and *recO* genes. An expanded view of the operon shows the *rncO* leader RNA (nucleotides +1 to +161) and the AUG translation start site. The red arrows indicate the RNase III cleavage sites.

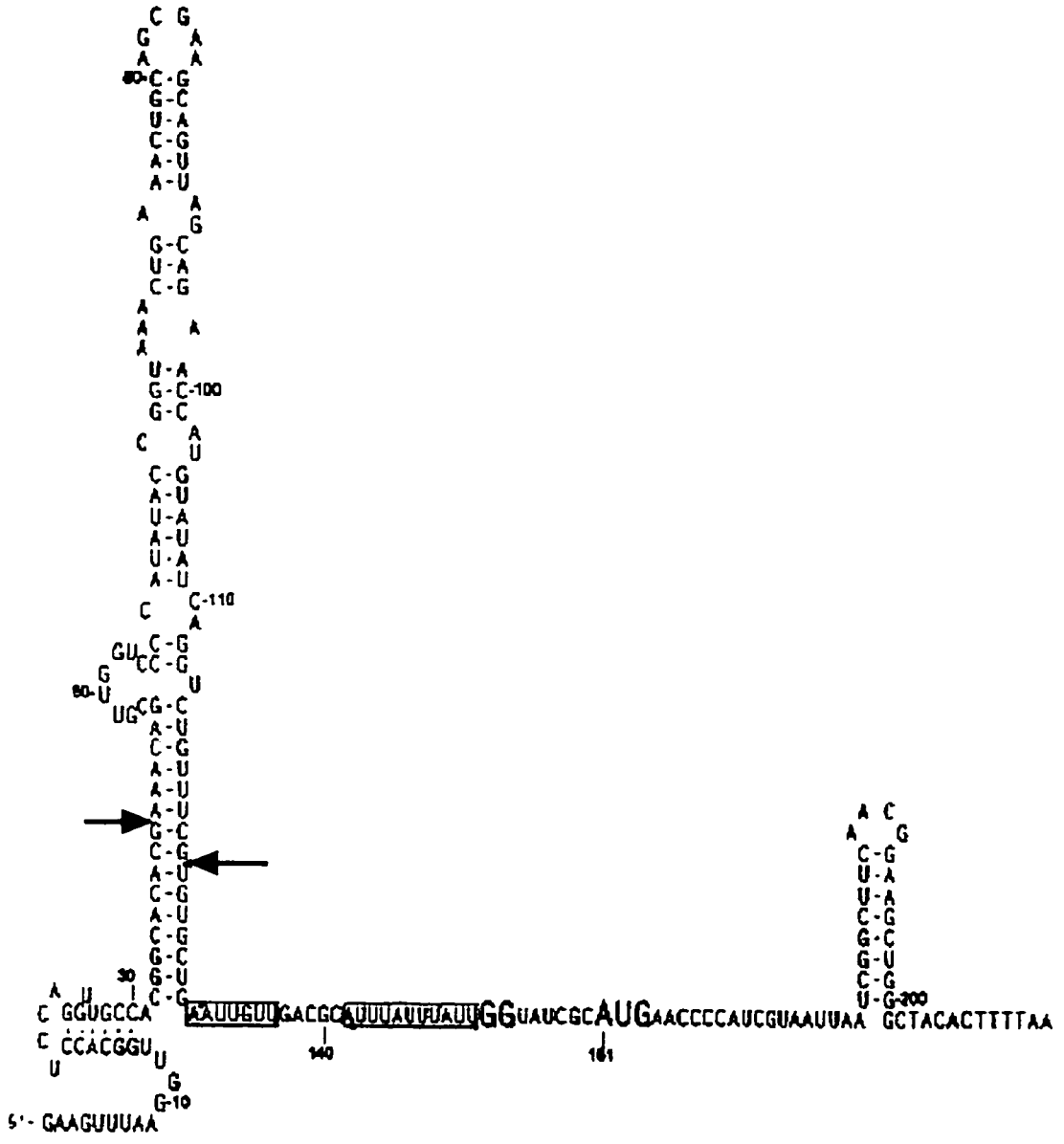


Figure 7. Sequence of the *rncO* leader RNA (adapted from Matsunaga *et al.*, 1996). The RNase III cleavage sites are indicated by the red arrows.

rnc operon contains a 5'-end-proximal "stabilizing sequence", termed the *rnc* operator (*rncO*) sequence (Figure 7). The *rncO* sequence is approximately 161 nucleotides (+1 to +161) and contains two stem-loop structures. Cleavage by RNase III within the second stem-loop destabilizes the mRNA transcript. Specifically, the cleaved *rnc* mRNA is a substrate for further degradation by RNase E (Matsunaga *et al.*, 1996).

Several other mRNAs undergo cleavage by RNase III within the 5'-leader sequence. Cleavage within these 5'-leader sequences also destabilizes the transcripts. How cleavage in the 5' region of these mRNAs leads to destabilization is not clear, but may be similar to the mechanism established for the *rnc* operon mRNA (see above). RNase III cleavage in the 5' leader could remove the ribosome binding site. Ribosomes would not bind to the cleaved mRNA, allowing the uncovered transcript to become susceptible to cleavage by degradative nucleases, such as RNase E (Cannistraro and Kennell, 1989). In principle, cleavage of the 5' leader might provide a binding site for a 5'→3' exoribonuclease. However, no such enzyme has been detected in bacterial cells (Belasco, 1993), although 5'→3' exoribonuclease activities exist in eukaryotic cells (Brawerman, 1993)

In contrast to the role in mRNA turnover, RNase III cleavage within the 5' leader of the *E. coli* alcohol dehydrogenase (Adh) mRNA is required for efficient translation (Aristarkhov *et al.*, 1996). Presumably, secondary structures within the Adh mRNA 5' leader obstructs the ribosome binding site. RNase III cleavage removes the occluding secondary structures, allowing ribosome binding. RNase III is required for *E. coli* growth in anaerobic conditions in which glucose or mannitol is the sole carbon/energy source (Aristarkhov *et al.*, 1996). Under these conditions, Adh is essential for cell growth.

RNase III cleavage can initiate degradation of specific viral mRNAs. Examples include the phage λ Integrase and *N* protein mRNAs (Wilder and Lozeron, 1979; Gottesman *et al.*, 1982; Guarneros *et al.*, 1982). RNase III cleavage of these mRNAs occurs at a 3'-end-proximal stem-loop structure (the Integrase mRNA RNase III cleavage

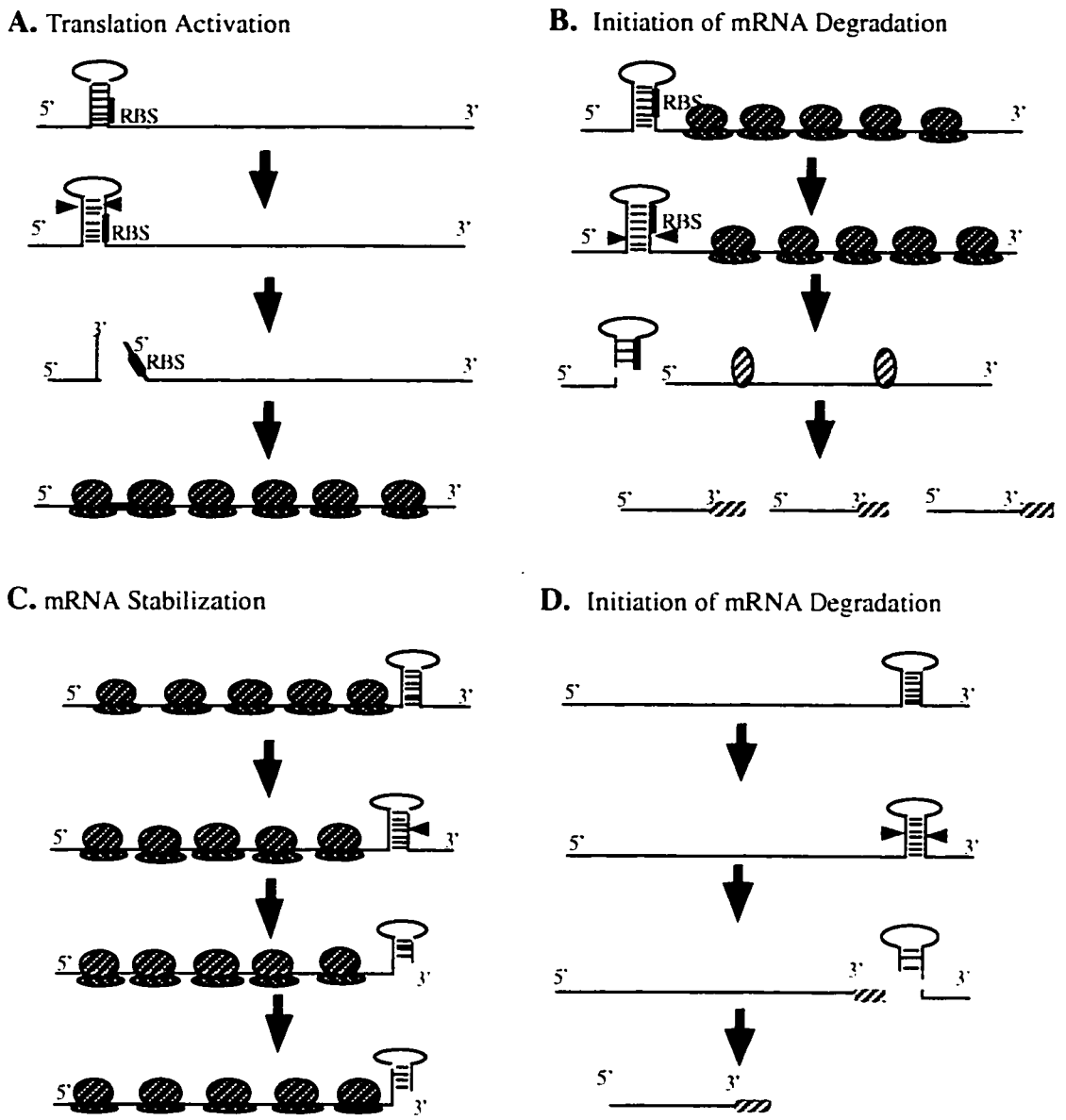
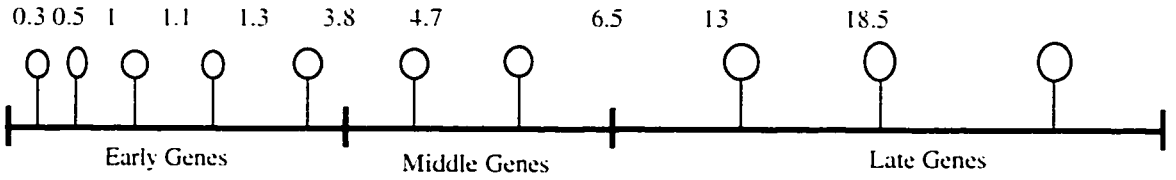


Figure 8. Functional consequences of RNase III cleavage of 5' and 3' stem-loop structures.
Panel A. Cleavage of 5'-UTR activates translation.
Panel B. Cleavage inactivates translation, and destabilizes mRNA transcripts.
Panel C. Cleavage of T7 polycistronic mRNAs creates 3'-hairpin stabilizers
Panel D. Cleavage of 3' stem-loops destabilizes mRNA transcripts.

= ribosome, = endoribonuclease, = 3'⇒5' exoribonuclease.
► = RNase III cleavage site. RBS=ribosome binding site

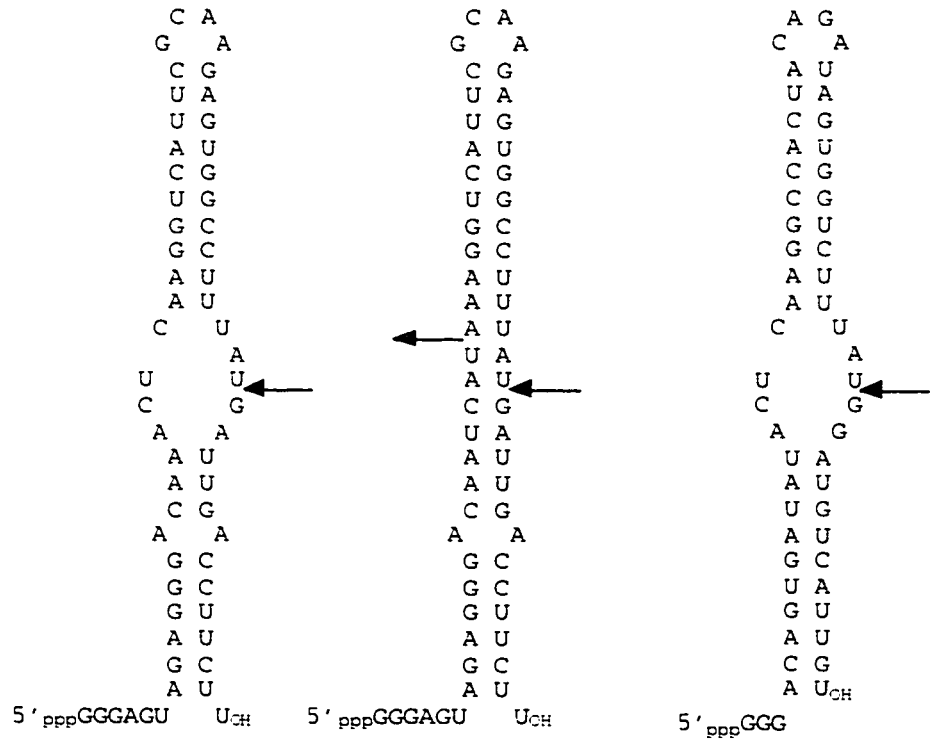
T7 genome



T7 RNase III processing sites	Nucleotide[a]	T7 units[b]
R0.3	890	2.2
R0.5	1468	3.7
R1	3138	7.9
R1.1	5887	14.7
R1.3	6448	16.2
R3.8	11,203	28.1
R4.7	13,892	34.8
R6.5	18,562	46.5
R13	27,280	68.3
R18.5	36,855	92.3

Figure 9. Positions of RNase III cleavage sites in the T7 genetic map. Diagram indicates stem-loop structures (♈) in T7 RNAs which are cleaved by RNase III. Numbers above ♈ indicate the T7 genes immediately upstream of the RNase III processing site.

[a] The last nucleotide at the 3' end of the upstream RNA sequence produced by cleavage.
[b] A T7 unit is 399.36 nucleotides.



T7 R1.1 **T7 R1.1[WC]** **T7 R18.5**

Figure 10. Sequences of bacteriophage T7 RNase III substrates. RNase III cleavage sites are indicated with red arrows

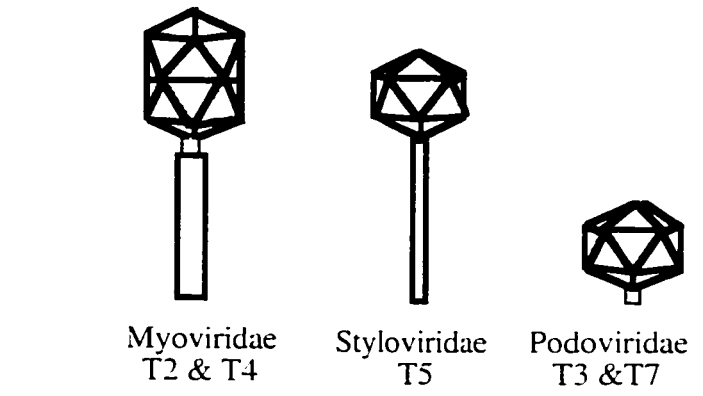
site is called the *sib* site). Cleavage removes the 3' stem-loop structure, creating an upstream RNA sequence susceptible to 3'→5' exoribonuclease digestion by RNase II and/or polynucleotide phosphorylase (Figure 8).

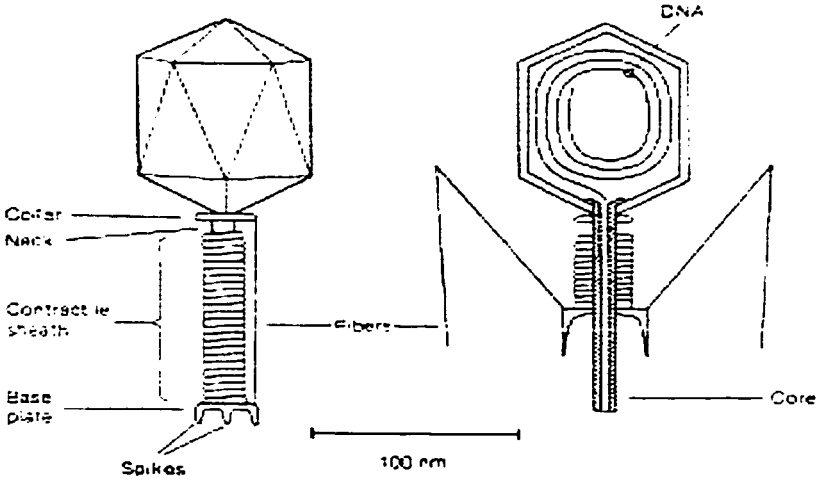
RNase III also site-specifically cleaves a number of bacteriophage T7 mRNAs (Figures 9 and 10). As discussed below, it is in the T7-infected cell that protein phosphorylation plays an important role in regulating RNase III activity and T7 RNA metabolism.

D. Bacteriophage T7.

RNase III is a key host enzyme in the strategy of infection of the bacteriophage T7. T7 is a lytic coliphage, belonging to the *Podoviridae* group (Figure 11). Phage T7 was initially isolated and included in the "T" phage collection of Delbrück and coworkers (Stent, 1963). The T-odd phages (T1, T3, and T7) possess short non-contractile tails and icosahedral heads. The T-even phages (T2, T4, and T6) and the T-odd phage T5 possess long contractile tails and elongated heads. The DNA sequence similarity between T2 and T4 is 85%, with the late genes exhibiting greater homology than the early genes. T1 is a highly persistent phage in the laboratory, being able to survive for years in a desiccated state, and can be a pernicious contaminant of bacterial cultures (Drexler and Christensen, 1961). For this reason very little is known about T1. T7 and the T3 are the smallest of the T phages and perhaps the best characterized. T7 and the T3 infect species of *Escherichia, Shigella, Salmonella, Klebsiella,* and *Pasteurella* (Hausmann, 1976).

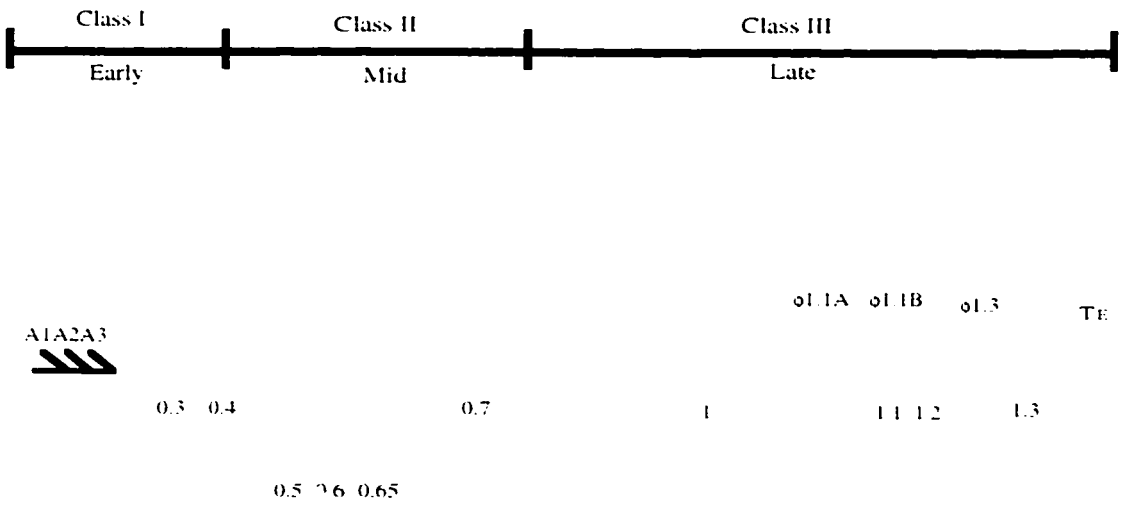
The T7 chromosome is a linear dsDNA of 39.936 bp, and encodes approximately 50 genes (Dunn and Studier, 1983). The T7 genes have been divided into three classes, based on their physical location in the genome, and mode and timing of transcription (Figures 12-14). The Class I genes are located at the "left end" of the chromosome and encode proteins that convert the infected host to an environment optimal for T7 reproduction (Figure 12).





T4

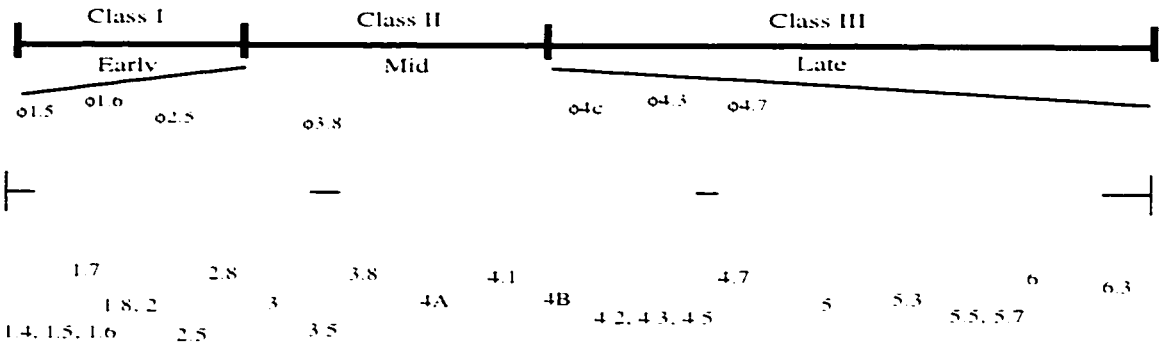
Figure 11. Structures of T-Group of phages



Gene	Amino Acids	M_r	Function
0.3	116	13.678	Inactivates host restriction enzymes
0.4	50	5621	?
0.5	47	4744	?
0.6A	53	6201	?
0.6B	111	13.250	?
0.7	359	41.124	Protein kinase
1	883	98.092	T7 RNA polymerase
1.1	42	5180	?
1.2	84	10.059	Replication
1.3	359	41.133	DNA ligase

Genetic Element	T7 DNA Nucleotide Position	Function
A1	498	*E. coli* RNAP promoter
A2	626	*E. coli* RNAP promoter
A3	750	*E. coli* RNAP promoter
ϕ 1.1A	5848	T7 RNAP promoter
ϕ 1.1B	5923	T7 RNAP promoter
ϕ 1.3	6409	T7 RNAP promoter
T_E	7588	*E. coli* RNAP terminator

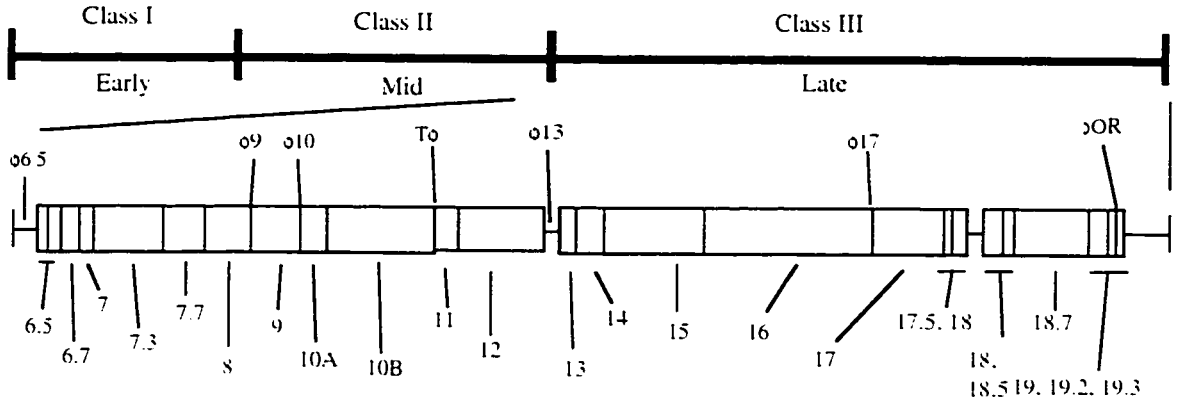
Figure 12. Bacteriophage T7 Class I genes and genetic elements.



Gene	Amino Acids	M_r	Function
1.4	51	5446	?
1.5	29	3174	?
1.6	86	9946	?
1.7	195	22053	?
1.8	48	5781	?
2	63	7043	Inactivates host RNA Polymerase
2.5	231	25.562	Single stranded DNA binding Protein
2.8	139	15.617	?
3	148	17.040	Endonuclease
3.5	150	16.806	Amidase (lysozyme)/T7 RNAP inhibitor
3.8	121	14.329	?
4A	566	62.656	Primase
4B	503	55.743	Primase
4.1	39	4265	?
4.2	112	12.653	?
4.3	70	7927	?
4.5	88	9960	?
4.7	135	15.208	?
5	704	79.692	DNA polymerase subunit
5.3	118	13.067	?
5.5	98	11.075	?
5.7	68	7280	Permits growth on lambda lysogens
6	347	39.995	Exonuclease
6.3	37	4088	?

Genetic Element	T7 DNA Nucleotide Position	Function
φ1.5	7778	T7 RNAP promoter
φ 1.6	7895	"
φ 2.5	9107	"
φ 3.8	11.180	"
φ 4A	12.671	"
φ 4.3	13.341	"
φ 4.7	13.915	"

Figure 13. Bacteriophage T7 Class II genes and genetic elements.



Gene	Amino Acids	M_r	Function
6.5	84	9474	?
6.7	87	9207	?
7	132	15.303	Host range
7.3	98	9937	Host range
7.7	130	14.737	?
8	535	58.989	Head-tail protein
9	306	33.766	Head assembly protein
10A	344	36.414	Major head protein
10B	397	41.800	Minor head protein
11	196	22.289	Tail protein
12	793	89.265	Tail protein
13	138	15.852	Internal virion protein
14	195	20.836	Internal virion protein
15	746	84.210	Internal virion protein
16	1318	143.840	Internal virion
17	552	61.441	Tail fiber protein
17. 5	67	7391	?
18	89	10.145	DNA maturation
18.5	143	16.243	?
18.7	82	9195	?
19	585	66.130	DNA maturation
19.2	84	9264	?
19.3	56	6429	?
19.5	49	5434	?

Genetic	T7 DNA Nucleotide Position	Function
φ 6.5	18.544	T7 RNAP promoter
φ 9	21.864	T7 RNAP promoter
φ 10	24.209	T7 RNAP terminator
φ 13	27.273	T7 RNAP promoter
φ 17	34.565	T7 RNAP promoter
φ OR	39.228	T7 RNAP promoter

Figure 14. Bacteriophage T7 Class III genes and genetic elements.

The Class II genes encode proteins involved in phage DNA synthesis and processing (Figure 13). The Class III genes are located on the "right end" of the chromosome and encode proteins involved in phage particle assembly and release from the host (Figure 14). The T7 genes are temporally expressed according to their map positions: the Class I genes are the early genes: the Class II genes are the middle genes: and the Class III genes are the late genes. (Studier, 1972, Hausmann, 1976; Dunn and Studier, 1981; Studier and Dunn, 1983).

Immediately following T7 adsorption and DNA injection, the host RNA polymerase transcribes the Class I genes from one of three strong promoters (A1, A2, and A3). A rho-independent transcription terminator (T_E) which immediately follows gene 1.3 prevents premature transcription of the Class II and Class III genes (Jeng *et al.*, 1992; Dunn and Studier, 1983). Expression of the Class I genes occurs until ~eight minutes post-infection. The T7 early gene 1 encodes a phage promoter-specific RNA polymerase (T7 RNA polymerase) which is responsible for transcribing the Class II and Class III genes (Figure 12) (Iost *et al.*, 1992; Macdonald *et al.*, 1993). The Class II genes are expressed from six to fifteen minutes post-infection. Of the nine known functions of Class II genes, six are directly involved in phage DNA replication (Figure 13). The Class III genes are expressed from eight to twenty-five minutes post-infection (i.e., up to cell lysis). The Class III gene products are responsible for phage maturation and assembly (Figure 14).

E. T7 Protein Kinase.

The T7 early gene 0.7 encodes an approximately 40 kDa protein kinase (T7 PK), containing 359 amino acids (Pai *et al.*, 1975a,b). The T7 PK contains two functional domains (Figure 15). The amino-terminal two-thirds of the T7 PK exhibits a protein kinase activity, while the carboxyl-terminal one-third exhibits a host transcription "shut-off" activity. The shut-off activity inhibits the action of the host RNA polymerase (Rothman-Denes *et al.*, 1973; Studier, 1973; McAllister and Barrett, 1977) by an unknown

mechanism. The two functional domains of the T7 PK can be uncoupled by mutation: T7 PK mutants have been isolated which selectively lack either the shut-off function or the protein kinase activity (Rothman-Denes *et al.*, 1973; Studier 1973, Michalewicz and Nicholson 1992).

The T7 PK is cAMP-independent and serine/threonine-specific (Pai *et al.*, 1975b). Over 90 proteins are phosphorylated following T7 PK expression in the infected cell (Robertson *et al.*, 1994). The identified targets include the translation initiation factors IF-1, IF-2, and IF-3, ribosomal proteins S1 and S6, elongation factors EF-G and EF-P, and the β' subunit of the host RNA polymerase (Robertson and Nicholson, 1992; Robertson *et al.*, 1994). Phosphorylation of IF-1, IF-2, and IF-3 causes a 2-3 fold decrease in fMet-tRNA binding to programmed 30S subunits *in vitro* (R.A. Provost and J.W.B. Hershey, personal communication). All three initiation factors are phosphorylated on threonine, while IF-2 is also phosphorylated on serine (Robertson and Nicholson, 1992).

Until recently there had been no evidence for bacterial translational control by protein phosphorylation. This is in contrast to eukaryotic cells, where translation is subject to phosphorylation control at multiple points (Hershey, 1989; Hershey, 1991; Zhou *et al.*, 1993). The discovery that the bacterial translational apparatus is covalently modified by a protein kinase, and that the functional activity of initiation factors is altered provides an important paradigm with which to understand the control of translation by covalent modification.

F. Involvement of T7 Protein Kinase in T7 RNA Processing by RNase III.

RNase III cleaves at five sites in the T7 Class I polycistronic mRNA precursor, generating the mature mono-, di- and tricistronic mRNAs. RNase III cleaves at two sites in the Class II (middle) transcripts, and three sites in the Class III (late) transcripts, generating the mature mRNAs (Figures 9 and 10) (Dunn and Studier, 1983). RNase III cleavage

```
1    MetAsnIleThrAspIleMetAsnAlaIleAspAlaIleLysAlaLeuProIleCysGluLeuAsp
1    ATGAACATTACCGACATCATGAACGCTATCGACGCAATCAAAGCACTGCCAATCTGTGAACTTGAC

23   LysArgGlnGlyMetLeuIleAspLeuLeuValGluMetValAsnSerGluThrCysAspGlyGlu
67   AAGCGTCAAGGTATGCTTATCGACTTACTGGTCGAGATGGTCAACAGCGAGACGTGTGATGGCGAG

45   LeuThrGluLeuAsnGlnAlaLeuGluHisGlnAspTrpTrpThrThrLeuLysCysLeuThrAla
133  CTAACCGAACTAAATCAGGCACTTGAGCATCAAGATTGGTGGACTACCTTGAAGTGTCTCACGGCT

67   AspAlaGlyPhetLysMeLeuGlyAsnGlyHisPheSerAlaAlaTyrSerHisProLeuLeuPro
199  GACGCAGGGTTCAAGATGCTCGGTAATGGTCACTTCTCGGCTGCTTATAGTCACCCGCTGCTACCT

89   AsnArgValIleLysValGlyPheLysLysGluAspSerGlyAlaAlaTyrThrAlaPheCysArg
265  AACAGAGTGATTAAGGTGGGCTTTAAGAAAGAGGATTCAGGCGCAGCCTATACCGCATTCTGCCGC

111  MetTyrGlnGlyArgProGlyIleProAsnValTyrAspValGlnArgHisAlaGlyCysTyrThr
331  ATGTATCAGGGTCGTCCTGGTATCCCTAACGTCTACGATGTACAGCGCCACGCTGGATGCTATACG

133  ValValLeuAspAlaLeuLysAspCysGluArgPheAsnAsnAspAlaHisTyrLysTyrAlaGlu
397  GTGGTACTTGACGCACTTAAGGATTGCGAGCGTTTCAACAATGATGCCCATTATAAATACGCTGAG

155  IleAlaSerAspIleIleAspCysAsnSerAspGluHisAspGluLeuThrGlyTrpAspGlyGlu
463  ATTGCAAGCGACATCATTGATTGCAATTCGGATGAGCATGATGAGTTAACTGGATGGGATGGTGAG

177  PheValGluThrCysLysLeuIleArgLysPhePheGluGlyIleAlaSerPheAspMetHisSer
529  TTTGTTGAAACTTGTAAACTAATCCGCAAGTTCTTTGAGGGCATCGCCTCATTCGACATGCATAGC

199  GlyAsnIleMetPheSerAsnGlyAspValProTyrIleThrAspProValSerPheSerGlnLys
595  GGGAACATCATGTTCTCAAATGGAGACGTACCATACATCACCGACCCGGTATCATTCTCGCAGAAG

221  LysAspGlyGlyAlaPheSerIleAspProGluGluLeuIleLysGluValGluGluValAlaArg
661  AAAGACGGTGGCGCATTCAGCATCGACCCTGAGGAACTCATCAAGGAAGTCGAGGAAGTCGCACGA
     → Shut-off domain
243  GlnLysGluIleAspArgAlaLysAlaArgLysGluArgHisGluGlyArgLeuGluAlaArgArg
727  CAGAAAGAAATTGACCGCGCTAAGGCCCGTAAAGAACGTCACGAGGGGCGCTTAGAGGCACGCAGA

265  PheLysArgArgAsnArgLysAlaArgLysAlaHisLysAlaLysArgGluArgMetLeuAlaAla
793  TTCAAACGTCGCAACCGCAAGGCACGTAAAGCACACAAAGCTAAGCGCGAAAGAATGCTTGCTGCG

287  TrpArgTrpAlaGluArgGlnGluArgArgAsnHisGluValAlaValAspValLeuGlyArgThr
859  TGGCGATGGGCTGAACGTCAAGAACGGAGTAACCATGAGGTAGCTGTAGATGTACTAGGAAGAACC


309  AsnAsnAlaMetLeuTrpValAsnMetPheSerGlyAspPheLysAlaLeuGluGluArgIleAla
925  AATAACGCTATGCTCTGGGTCAACATGTTCTCTGGGGACTTTAAGGCGCTTGAGGAACGAATCGCG

331  LeuHisTrpArgAsnAlaAspArgMetAlaIleAlaAsnGlyLeuThrLeuAsnIleAspLysGln
991  CTGCACTGGCGTAATGCTGACCGGATGGCTATCGCTAATGGTCTTACGCTCAACATTGATAAGCAA

353  LeuAspAlaMetLeuMetGly
1057 CTTGACGCAATGTTAATGGGC
```

enhances translation of several T7 mRNAs and provides 3'-hairpin structures which protect the mRNAs from 3'→5' exonucleolytic action (Dunn and Studier, 1983).

In 1983, Mayer and Schweiger reported a four-fold increase in RNase III activity in extracts of *E. coli* cells infected by T7 (Mayer and Schweiger, 1983). The study used as substrate a synthetic double-stranded RNA [poly r(A)·poly r(U)] to assay the activity of RNase III. It was found that expression of the T7 protein kinase (T7 PK) in the infected cell induces the phosphorylation of RNase III ~4-6 minutes post-infection (37^{0}C), and that phosphate is transferred to one or more serine residues. However, the following issues remain unresolved: First, since these experiments used extracts of T7-infected cells, it was not determined whether the T7 PK directly phosphorylates RNase III, or whether there is a secondary (downstream) protein kinase involved. Second, although the phosphoamino acid is serine in RNase III, as isolated from T7-infected cells (Mayer and Schweiger, 1983; Robertson *et al.*, 1994), the location(s) of the target serine(s) in the primary sequence is not known. Third, it is not known whether phosphorylation is directly responsible for the increase in RNase III activity, or whether there is a separate interaction of RNase III with T7 PK which mediates the stimulation. Fourth, it is not known whether the increase in RNase III activity is also observed with natural cellular and viral RNase III substrates; and if so, whether there is preferential enhancement of processing of T7 substrates. Fifth, it is not known whether RNase III phosphorylation alters substrate cleavage site choice. Finally, is the T7 PK-dependent increase in RNase III processing activity due to enhancement of substrate binding, or cleavage, or both?

G. Specific aims and objectives

The specific aim of my dissertation research has been to determine how the T7 protein kinase regulates the RNA processing activity of *E. coli* ribonuclease III.

The specific objectives are to: **1)** determine whether RNase III is a substrate for T7 protein kinase: **2)** establish conditions for the optimal phosphorylation of RNase III *in vitro:* **3)** identify phosphorylation site(s) in RNase III: **4)** create RNase III mutants which are resistant to phosphorylation by the T7 protein kinase: **5)** determine how phosphorylation stimulates RNase III activity (i.e., whether the increased processing activity is due to a K_M (substrate binding) effect, a k_{cat} (chemical step) effect, or both): and **6)** determine whether there is substrate preference for phosphorylated RNase III.

MATERIALS AND METHODS

A. Chemicals and reagents

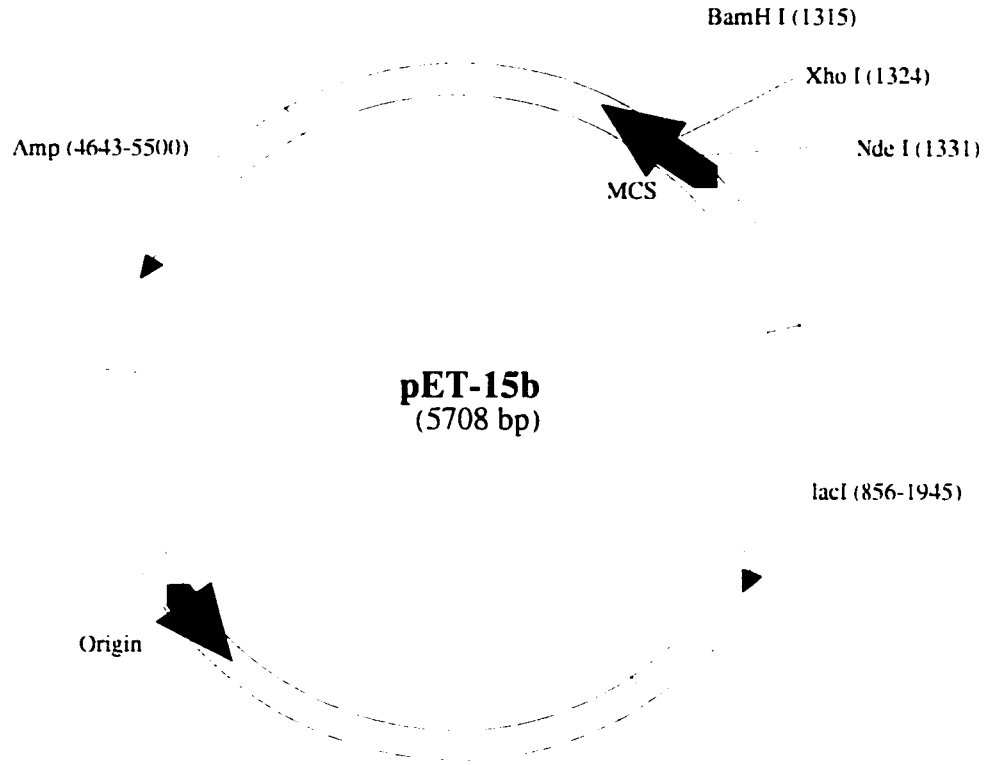
Chemicals were molecular biology grade or better, and were obtained from Fisher Scientific (Chicago, IL) or Sigma Chemical Co. (St. Louis, MO). Bacterial growth media was purchased from Difco (Detroit, MI). Restriction enzymes, T4 DNA ligase, Vent DNA polymerase, T4 polynucleotide kinase, and bacteriophage lambda protein phosphatase were purchased from New England Biolabs (Beverly, MA) or Promega (Madison, WI). Maxi plasmid purification kits, dNTPs, and rNTPs were purchased from Promega. Calf intestine alkaline phosphatase was purchased from Boehringer-Mannheim (Indianapolis, IN). DNA sequencing kits were purchased from Amersham (Arlington Heights, IL). Agarose was purchased from GIBCO-BRL (Gaithersburg, MD). His-bind resin for protein purification, and pET plasmids were purchased from Novagen (Madison, WI). Spectrapore dialysis tubing was purchased from Fisher Scientific (Itasca, IL). T7 RNA polymerase was purified in-house from the overexpressing *E. coli* strain (BL21/ pAR1219), according to the procedure of Grodberg and Dunn (1988). DNA oligonucleotides and PCR primers were synthesized by the Wayne State University Biological Sciences Core Facility. Radioactive materials ([α-^{32}P]UTP (3000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), ^{32}P-orthophosphate (8500 Ci/mmol), and [^{35}S-Met,^{35}S-Cys] protein labeling mix (1000 Ci/mmol)) were purchased from Dupont-NEN (Boston, MA). IEF ampholytes (pH 5-7 and pH 3-10) were purchased from Pharmacia-LKB (Piscataway, NJ). Photographic chemicals were purchased from Kodak (Rochester, NY). X-ray film was either Fuji or Kodak. Protein assay reagents were purchased from Bio-Rad (Hercules, CA).

B. Bacterial strains

E. coli strains used in experiments were: BL21 [*ompT* r^-_B m^-_B]; BL21(DE3) [*ompT* r^-_B m^-_B λ (DE3)], HMS174 [*rec*A r^-_K m^+_K rif^r], HMS174(DE3) [*rec*A r^-_K m^+_K rif^r (DE3)]; W3110 (K12); and DH5α[*rel*A *rec*A r^-_K m^+_K]. Recombinant pET plasmids used included: pET-15b(PK[JS78]), which contains a truncated version of the T7 protein kinase gene, with the C-terminus of the protein kinase established by a point mutation that changes Gln243 to an amber codon (Michalewicz and Nicholson, 1992); pET-15b(*rnc*), encoding the RNase III gene (Li *et al.*, 1993), pET-15b(*dsRBD*), encoding the RNase III dsRBD; and pET-15b(*catalytic domain*), encoding the RNase III catalytic domain. Various mutant RNase III, dsRBD, and T7PK genes were also expressed from recombinant pET plasmids (see **Site-directed mutagenesis** below).

C. T7 phage strains

T7 and T7 (PK^-) phage stocks were obtained from F.W. Studier (Brookhaven National Laboratory). T7 phage were grown on W3110 and purified by CsCl centrifugation as described (Studier, 1969). Phage titers were determined by plating phage dilutions on W3110 lawns (Studier, 1969). Phage stocks were stored at 4°C in CsCl.



Bgl II T7 promoter lac operator *Xba* I

AGATCTCGGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATA

RBS His-tag

TTTTGTTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCC

Nde I *Xho* I *Bam* HI

GCGGCGGCAGCCATATGCTCGAGGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCT

T7 terminator

GAGCAATAACTAGCATAACCCCTTGGGGCCTCAAACGGGTCTTGAGGGGTTTTTTG

Figure 16. The pET-15b plasmid and genetic elements. The pET-15b vector encodes the T7ϕ10 promoter, *lac* operator, and an N-terminal His-tag sequence followed by three cloning sites (*Nde* I, *Xho* I, and *Bam* HI). The location of the ampicillin resistance gene (Amp), origin of replication, the multiple cloning site (MCS), and the lactose repressor gene (lacI) are also indicated.

D. Molecular cloning

The pET plasmids are useful vectors for the controlled overexpression of recombinant proteins (Figures 16 and 17) (Studier and Moffatt, 1985; Studier *et al.*, 1990). This system was used to express and purify the T7 protein kinase, RNase III, the RNase III dsRBD, the RNase III catalytic domain, and specific mutant versions of these polypeptides.

The target genes are cloned into the pET vector multiple cloning site immediately downstream from the T7φ10 promoter, allowing T7 RNA polymerase to direct target genemRNA synthesis (the T7φ10 promoter is not recognized by the host RNA polymerase). To overexpress the target protein, the recombinant plasmid is placed in a host which has the T7 RNA polymerase gene integrated into the chromosome *via* the DE3 lambda phage (Studier *et al.*, 1990). The T7 RNA polymerase gene is under control of the inducible *lacUV5* promoter (Figure 17). Addition of IPTG to the growing culture induces the production of T7 RNA polymerase, which then directs transcription from the T7φ10 promoter (Studier and Moffatt, 1985). In addition, IPTG abolishes lactose repressor protein binding to the *lac* operator, which is immediately downstream of the T7φ10 promoter. The specific gene or gene fragment was cloned between the *Nde* I and *Bam* HI sites of pET-15b, such that a hexahistidine sequence is fused to the N-terminus of the expressed protein.

Recombinant pET-15b(*rnc*) and pET-15b(*dsRBD*) plasmids were provided by A. Amarasinghe. The recombinant pET-15b(*catalytic domain*) plasmid was provided by E-J. Jun. The recombinant pET-15b(*rnc* [E117K]) plasmid was provided by H. Li. Recombinant pET-15b(*PK* [JS78]) and pET-15b(*PK* [G76F]) plasmids were provided by P. Sharma.

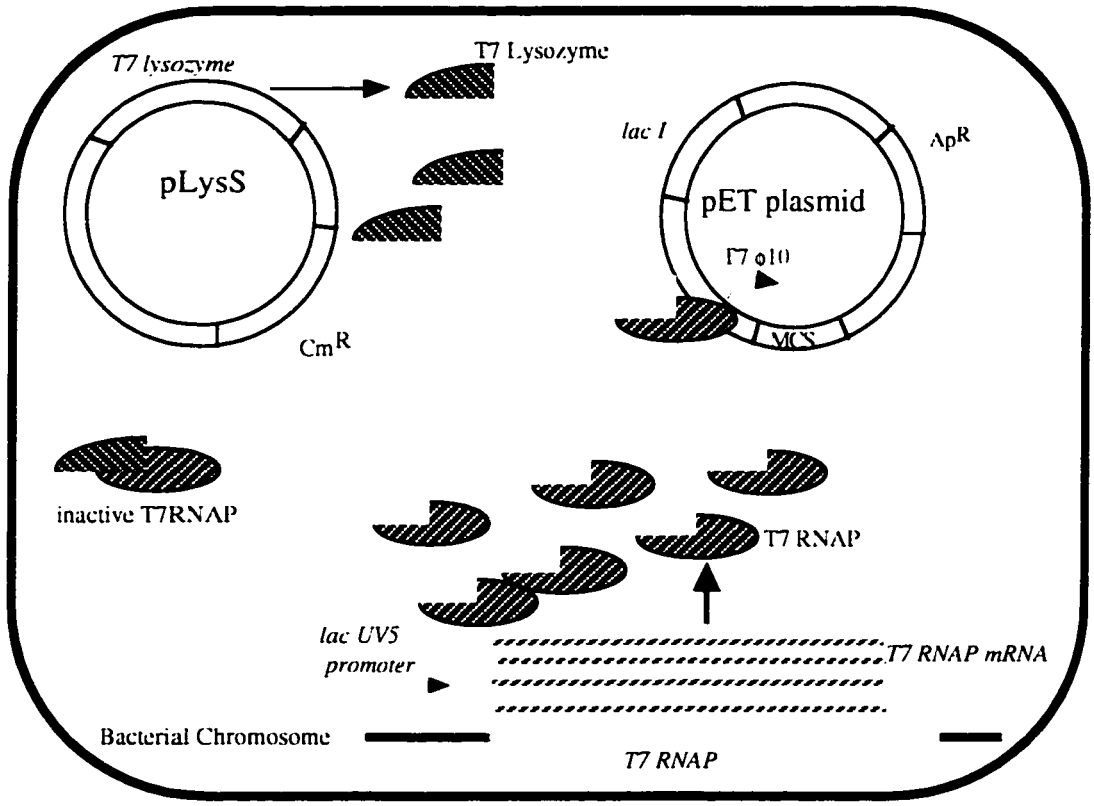


Figure 17. Schematic representation of pET expression system (see **Molecular Cloning**). Interior of *Escherichia coli* depicting expression from the pLysS and pET plasmids. The lactose repressor binds to the *lacUV5* promoter and prevents of basal-level expression of the T7 RNA polymerase (RNAP). The pLysS plasmid also suppresses basal-level expression of T7 RNAP: the T7 lysozyme binds to and inactivates T7 RNAP. The addition of 1 mM IPTG to the growing culture inactivates the lactose repressor protein allowing synthesis by T7 RNAP. The T7 RNAP in turn directs transcription through the MCS from the T7φ10 promoter. Abbreviations are: *T7 RNAP*, T7 RNA polymerase gene; T7φ10, T7 specific promoter for T7 RNAP; *lacUV5*, IPTG inducible promoter; *lacI*, lactose repressor gene; MCS, multiple cloning site

Table 4. List of Proteins Used in this Study.

Protein	Amino acid change(s)	Biochemical Phenotype
RNase III	---	(wt)
RNase III	E117K	Binds substrate; unable to cleave substrate
RNase III	S195A.S198A	Phosphorylation-resistant dsRBD
dsRBD	---	(wt)
dsRBD	S195A	dsRBD unable to be phosphorylated by T7 PK
dsRBD	S198A	dsRBD unable to be phosphorylated by T7 PK
dsRBD	S195A.S198A	dsRBD unable to be phosphorylated by T7 PK
dsRBD	S206A	---
dsRBD	S207A	---
dsRBD	S206A.S207A	---
Catalytic domain	---	Cleaves substrate in low salt. Mn^{+2}
T7 PK(JS78)	---	wt phosphotransferase
T7 PK(JS78)	G76F	unable to transfer phosphate

E. Site-directed mutagenesis of the RNase III dsRBD and RNase III

Site-directed mutagenesis of the *rnc* gene and RNase III *dsRBD* sequence was performed using PCR, essentially as described by Landt and coworkers (Vandeyar *et al.*, 1988; Landt *et al.*, 1990). The four serine codons within the RNase III dsRBD [Ser195, Ser198, Ser207, Ser208] (see Figure 2) were mutated to alanine codons, either singly or in combination (Table 4). The resulting *dsRBD* fragments encoding the mutations were digested with *Nde* I and *Bam* HI and cloned into the *Nde* I and *Bam* HI sites of pET-15b. Sequence analysis of the recombinant plasmids verified correct incorporation of mutations, and the absence of adventitious mutations. Serines 195 and 198 within RNase III were mutated to alanine codons *via* the same mutagenesis protocol, and the PCR product was digested with *Nde* I and *Bam* HI and cloned into the *Nde* I and *Bam* HI sites of pET-15b.

F. Protein overexpression and purification

The Histidine-Tag (His-Tag) protein purification system (Figures 18 and 19) (Chen and Hai, 1994), was used for the purification of T7 PK, RNase III, the RNase III dsRBD, the RNase III catalytic domain, and specific mutants of RNase III, the dsRBD, and T7 PK (Table 4). The *E. coli* DE3 lysogen containing the recombinant pET-15b plasmid is grown at 37^{0}C in LB + Ampicillin (50 μg/ml) and treated with 1 mM IPTG during early log phase. Two hours later (the following steps were carried out at 4^{0}C) the cells are harvested and lysed by sonication in Binding buffer (see **List of Buffers and Solutions**). The cell lysate is centrifuged to remove debris, and the supernatant directly applied to a His-Bind affinity column (2.5 ml). The N-terminal hexahistidine sequence binds to Ni^{+2} ions which are immobilized on a metal chelation column. After repeated washing to remove unbound protein and nucleic acid, the His-tagged protein is eluted using a buffer containing 1 M imidazole. The imidizole displaces the His-tagged protein from the Ni^{+2} ions. The eluted protein is placed into dialysis tubing (molecular weight cut-off ~3500) and dialyzed against

the appropriate buffers (see **List of buffers and solutions**). Specifically, each protein sample was dialyzed against the appropriate storage buffer #1 (1:4000 dilution factor) for 12 hours, then dialyzed against the appropriate storage buffer #2 (1:4000 dilution factor) for 12 hours. Glycerol was added to the sample (final concentration of 50%) and stored at -20°C. Protein concentrations were determined by a Bio-Rad protein assay.

It was suspected that during the overexpression and purification steps, the T7 PK (and perhaps also the T7 PK[G76F] mutant) may have undergone autophosphorylation (or phosphorylation by another protein kinase) and therefore contain phosphoserine residues. Thus, after purification, the His-T7 PK and His-T7 PK[G76F] were dialyzed against Binding buffer for 12 hours. Approximately 1 mg of each protein was treated with 200 units of λ protein phosphatase for 2 hours at 37°C. An additional 200 units of λ protein phosphatase was added followed by incubation for 2 hours at 37°C. The reaction was loaded onto a fresh His-bind column, and the proteins recovered by elution with 1 M imidizole, and treated as described above.

The concentrations of the purified proteins were: His-RNase III (0.1 mg/ml); His-dsRBD (1 mg/ml); His-dsRBD[S195A] (1 mg/ml); His-dsRBD[S198A] (1 mg/ml); His-dsRBD[S206A] (0.9 mg/ml); His-dsRBD[S207A] (1 mg/ml); His-dsRBD[S206A,S207A] (1.2 mg/ml); His-catalytic domain (2 mg/ml); His-RNase III[E117K] (0.1 mg/ml); His-RNase III[S195A,S198A] (1 mg/ml); His-T7 PK (1 mg/ml); and His-T7 PK[G76F] (1 mg/ml).

G. *In vitro* phosphorylation of protein by T7 protein kinase

The method used for the *in vitro* [^{32}P] phosphorylation of protein by T7 PK is based on a previously described protocol, with several modifications (Pai *et al.*, 1975a,b). The reaction mixture contains: 25 mM Tris-HCl (pH 7.0), 15 mM $MgCl_2$, 0.1 mM EDTA, 10 μCi [γ-^{32}P] ATP (3000 mCi/mmol), ~1 μg of substrate (e.g., RNase III) and 1 μg of T7 PK. The reactions are initiated by adding $MgCl_2$, followed by incubation at 30°C. The

precise order of addition of components to the phosphorylation reaction is essential for maximizing the phosphorylation efficiency (see **Results**). Reactions were stopped by adding EDTA (20 mM final concentration). One-tenth volume of SDS-PAGE sample buffer is added, the samples boiled for 3 minutes, and aliquots electrophoresed in an SDS-polyacrylamide gel. The gels are covered with plastic wrap, and the amount of incorporated ^{32}P-radioactivity determined by radioanalytic imaging (AMBIS, 1.5% counting efficiency) (Li *et al.*, 1993). The gels are also exposed to x-ray film at -70^{0}C using intensifying screens.

H. Preparation of phosphorylated RNase III

To prepare phosphorylated RNase III for use in substrate cleavage and binding assays, protein phosphorylation reactions were performed using an excess of nonradioactive ATP. The reactions were performed in the same manner as ^{32}P-phosphorylation reactions (see above) with the following changes: the ATP concentration was 1 mM. Following incubation at 30^{0}C, the sample was placed on ice until its use in subsequent cleavage and binding assays.

To calculate the level of RNase III phosphorylation in the non-radioactive reaction, radioactive protein kinase reactions were performed and supplemented with 1 mM rATP. The samples were electrophoresed in an SDS polyacrylamide gel and analyzed by radioanalytic imaging (1.5% counting efficiency), and the amount of incorporated phosphate calculated. The gel analysis results indicate that 15-20% of RNase III, RNase III[E117K], and RNase III[S195A,S198A] are phosphorylated.

I. *In vivo* radioisotopic (^{32}P, ^{35}S) labeling of T7-infected cell protein

E. coli W3110 cells were grown in modified MOPS minimal media (Neidhardt *et al.*, 1974) (~25 ml) at 30^{0}C with aeration to a density of approximately 5x10^8 cells/ml. The

cells are then infected with purified T7 phage (m.o.i. of ~10). At 0, 5, 10, 15, and 20 minutes post-infection, 1 ml aliquots are removed and incubated with [^{32}P] orthophosphate (50 μCi/ml final concentration) and [^{35}S] protein labeling mix (25 μCi/ml final concentration) for 5 minutes. The incorporation of radiolabel was stopped with cyanide-phosphate buffer (Studier, 1975), and the cells pelleted, resuspended in SDS-PAGE sample buffer, and boiled for 3 minutes. After cooling, the cell lysate is treated with a DNase/RNase mixture (Desmarquets *et al.*, 1984; Robertson *et al.*, 1994) and aliquots electrophoresed in an SDS polyacrylamide gel (see below). The gels are covered with plastic wrap, and differential autoradiography performed using x-ray film and intensifying screens at -70^{0}C. This technique provides two autoradiograms of each gel: a ^{32}P-specific autoradiogram, and an ^{35}S-specific autoradiogram (Cooper and Burgess, 1982).

J. SDS-polyacrylamide gel electrophoresis of protein

Proteins were analyzed by one-dimensional discontinuous SDS-PAGE (Laemmli 1970; Studier, 1973). A 12% or 15% polyacrylamide separation gel (1.5mm x 12cm x 12cm) was prepared and allowed to polymerize for one hour. A stacking gel (~4 cm) was poured and allowed to polymerize for one hour. SDS-PAGE sample loading buffer was added to the protein samples, followed by heating at 100^{0}C for three minutes. Samples were added to the gel wells, and electrophoresed at 30 mA (constant current) for six hours. The gels were either silver stained (Merril *et al.*, 1979; Switzer *et al.*, 1979), or stained with Coomassie Brilliant Blue R-250 (Meyer and Lamberts, 1965). If the gels contained radiolabeled protein, they were covered with plastic wrap, placed in X-ray film cassettes, and autoradiography performed using intensifying screens at -70^{0}C.

K. 2-Dimensional polyacrylamide gel electrophoresis of protein

The 2-dimensional gel electrophoresis system is based on the original O'Farrell method (O'Farrell, 1975; O'Farrell *et al.*, 1977), with modifications as described by R. A.

van Bogelen (van Bogelen *et al.*, 1990). Following electrophoresis the gels are dried and differential autoradiography performed as described (Cooper and Burgess, 1982). Analysis of protein phosphorylation patterns was performed using the standard *E. coli* Gene-Protein Index (Neidhardt *et al.*, 1989; van Bogelen *et al.*, 1990) as a reference pattern.

L. Synthesis and purification of RNase III substrates

The ^{32}P -labeled RNA substrates used in this study were enzymatically synthesized *in vitro* as described (Milligan *et al.*, 1987; Chelladurai *et al.*, 1991) with some modification. DNA oligonucleotides encoding the RNase III substrate were synthesized by the Wayne State University Biological Sciences Core Facility. The deprotected oligonucleotides were purified by electrophoresis in 15% polyacrylamide gels containing TBE and 7 M urea. The oligonucleotides were located in the gel by UV shadowing, then excised with a sterile scalpel. Oligonucleotides were recovered from the crushed gel slice by phenol extraction, followed by ethanol precipitation. To synthesize RNA, the purified oligonucleotides were annealed to an 18-nt promoter oligonucleotide to provide the T7ϕ10 promoter sequence in the required double-stranded form (Milligan *et al.*, 1987). The primer-template complex is combined with 1 mM each of the 4 rNTPS, 10 μCi of [α-^{32}P]UTP (3000 Ci/mmol), and T7 RNA polymerase (400 units) in reaction buffer (Milligan *et al.*, 1987) and incubated for at least 2 hours at 37^{o}C. Stop buffer (containing 20 mM EDTA, bromophenol blue, and xylene cyanol) (Chelladurai *et al.*, 1993) is added and the sample electrophoresed for four hours (20 V/cm constant voltage) in a 15% polyacrylamide gel containing 7 M urea in TBE buffer. The RNA is located in the gel by autoradiography, then excised with a sterile scalpel. The RNA is recovered from the crushed gel slice by phenol extraction, followed by ethanol precipitation (Chelladuri *et al.*, 1993).

Nonradioactive RNA was enzymatically synthesized and purified in the same manner as described above. The RNA was dephosphorylated by treatment with calf intestine alkaline phosphatase (CAPase) in phosphatase buffer for 1 hour at 37^{0}C. Typically, the 30 µl reaction contained ~200 pmol of RNA and 2 units of CAPase. The RNA was phenol extracted, ethanol precipitated, and resuspended in TE buffer. Approximately 50 pmols of dephosphorylated RNA was 5'-^{32}P-labeled using 10 units of T4 polynucleotide kinase and 20 µCi of [γ-^{32}P]ATP (3000 Ci/mmol). The reaction is stopped by adding an equal volume of gel loading buffer, then electrophoresed in a 15% polyacrylamide gel containing 7 M urea in TBE. The 5'-^{32}P-labeled RNA was located by autoradiography, phenol extracted, and ethanol precipitated.

The *rncO* RNA leader sequence was directly transcribed from a PCR product of plasmid pRS1398 (gift from Robert W. Simons). The pRS1398 plasmid contains the *rnc* operon with the *rncO* RNA leader sequence. Amplification of *rncO* RNA leader sequence was achieved with the use of flanking primers and Vent DNA polymerase, resulting in the creation of a 215 bp product. A 30 cycle PCR protocol was used with the following steps: step 1, 94^{0}C 2 minutes; step 2, 55^{0}C 1 minute; step 3, 72^{0}C 1 minute. The PCR product was gel purified in the same buffer as described in Milligan *et al.*, 1987. The *rncO* RNA leader was synthesized *in vitro* using T7 RNA polymerase (as described above except that 3 mM of rNTPS was used, and 30 µCi of [α-^{32}P]UTP (3000 Ci/mmol)). Use of a primer during the enzymatic synthesis was not necessary since the DNA template contains the T7 RNA polymerase promoter in double-stranded form. The RNA product was purified by gel electrophoresis as described above.

M. *In vitro* RNase III processing assay

RNase III substrate cleavage assays are performed in "physiological salt" reaction buffer which contains: 250 mM potassium glutamate, 10 mM $MgCl_2$, 30 mM Tris-HCl

(pH 7.5), 5 mM spermidine, 0.1 mM DTT, 0.1 mM EDTA, and 0.4 μg/ml purified *E. coli* tRNA (Li *et al.*, 1993). RNase III is combined with ^{32}P-labeled RNA (heated for 3 minutes at 85^{0}C and snap-cooled on ice) in Mg^{+2}-free reaction buffer on ice. The tubes are placed in a 37^{0}C water bath for one minute, and the reaction initiated by adding $MgCl_2$ (final concentration, 10 mM). Following incubation at 37^{0}C, the reactions are terminated by the addition of stop mix (containing 20 mM EDTA, bromophenol blue, and xylene cyanol) (Chelladurai *et al.*, 1993) and electrophoresed (20 V/cm constant voltage) in a 15% polyacrylamide gel containing TBE and 7 M urea. Autoradiography was performed using intensifying screens at -70^{0}C. Quantitation of the cleavage reactions was accomplished by radioanalytic imaging (Li *et al.*, 1993).

N. *In vitro* RNase III binding assay

Gel electrophoretic mobility shift assays were performed essentially as described (Chelladurai *et al.*, 1993; Li and Nicholson, 1996). It was necessary to use the RNase III[E117K] mutant in this assay, due to carryover of Mg^{+2} from the prior phosphorylation reaction: Mg^{+2} from this source was sufficient to support RNase III cleavage of substrate (data not shown), which prevented accurate determination of K_D values. Since RNase III[E117K] lacks cleavage activity but is still able to bind substrate (Li and Nicholson, 1996) the presence of Mg^{+2} in the binding reactions is irrelevant. To prepare ^{32}P-labeled RNA for the gel shift assay, any intermolecular RNA complexes which may have formed upon storage at -20^{0}C were disrupted by heating the 5'-^{32}P-labeled RNA at 90^{0}C for 30 seconds in TE buffer, then snap-cooled on ice. The RNA is combined with varying amounts of RNase III[E117K] mutant in binding assay buffer on ice [the binding assay buffer is the same as RNase III cleavage assay buffer (see **List of buffers and solutions**) except that the 10 mM $MgCl_2$ is replaced with 10 mM $CaCl_2$, and the buffer is supplemented with 1% (v/v) glycerol]. Samples are incubated at 37^{0}C for 10 minutes.

placed on ice for 20 minutes, then electrophoresed (10 V/cm constant voltage) at 5-6°C in an 8% polyacrylamide gel containing 0.5x TBE buffer and 10 mM $CaCl_2$. Autoradiograms were obtained using intensifying screens. The amounts of free and bound RNA were determined by radioanalytic imaging (Li and Nicholson, 1993).

O. Growth media, buffers, and solutions

All growth media, buffers, and solutions are described as working concentrations. All solutions were prepared with deionized and distilled H_2O.

Bacterial Growth Media

LB Broth (per liter)

10g	Bactotryptone
5g	Yeast Extract
10g	NaCl

LB Agar (per liter)

10g	Bactotryptone
5g	Yeast Extract
10g	NaCl
15g	Bacto-agar

Modified MOPS Minimal Media (pH 7.6)

40 mM	MOPS (pH 7.6)
4 mM	Tricine (pH 7.6)
0.01 mM	$FeCl_3$
9.5 mM	NH_4Cl
0.1 mM	$CaCl_2$
0.53 mM	$MgCl_2$
50 mM	NaCl
1.32 mM	K_2HPO_4
19.8 mM	Glucose
40 nM	H_3BO_3
3 nM	$CoCl_2$
1 nM	$CuSO_4$
8 nM	$MnCl_2$
1 nM	$ZnCl_2$

Filter sterilized

General Buffers and Solutions

TE Buffer (pH 7.0)
10 mM Tris-HCl
1 mM $EDTA\text{-}Na_2$

TBE Buffer (pH 8.0)
89 mM Tris base
89 mM Boric Acid
1 mM $EDTA\text{-}Na_2$

Ethidium Bromide
10 mg/ml Ethidium Bromide
Filter sterilized

Ampicillin
100 mg/ml ampicillin
Filter sterilized

Chloramphenicol (prepared in 95% Ethanol)
25 mg/ml Chloramphenicol
Filter sterilized

IPTG
1M IPTG
Filter sterilized

Virus Buffer
0.5M NaCl
5mM Tris-HCl (pH 7.6)
1mM $EDTA\text{-}Na_2$

Cyanide-Phosphate Buffer
30 mM Na_2HPO_4 (pH 7.2)
20 mM NaCN

T7 RNA Polymerase Transcription Buffer
40 mM Tris-HCl (pH 8.0)
20 mM $MgCl_2$
1 mM Spermidine
5 mM DTT
0.01% Triton X-100
80 mg/ml PEG 8000

RNase A Solution
2 mg/ml RNase A
0.1 M Sodium Acetate (pH 4.8)
0.3 M $EDTA\text{-}Na_2$
(heat treated for 10 minutes at 80^0C)

RNA Extraction Buffer
0.5 M Ammonium Acetate (pH 7.0)
10 mM $EDTA\text{-}Na_2$

DNase I Solution

2 mg/ml	DNase I
10 mM	HEPES (pH 7.5)
50%	Glycerol (v/v)
10 mM	$CaCl_2$
10 mM	$MgCl_2$

RNase III Cleavage Assay Buffer

30 mM	Tris-HCl (pH 7.5)
250 mM	Potassium Glutamate
10 mM	$MgCl_2$
5 mM	Spermidine
0.1 mM	DTT
0.1 mM	EDTA-Na_2
0.4 µg/ml	*E. coli* tRNA

RNase III Binding Assay Buffer

30 mM	Tris-HCl (pH 7.5)
250 mM	Potassium Glutamate
10 mM	$CaCl_2$
5 mM	Spermidine
0.1 mM	DTT
0.1 mM	EDTA-Na_2
0.4 µg/ml	*E. coli* tRNA
1%	Glycerol (v/v)

Protein Kinase Assay Buffer

30 mM	Tris-HCl (pH 7.2)
2 mM	NH_4Cl
1 mM	DTT
0.1 mM	EDTA-Na_2

30% Polyacrylamide Gel Solution (36.5:1 Acrylamide:Bis-acrylamide) (Cleavage assays)

29.2%	Acrylamide (w/v)
0.8%	Bis-acrylamide (w/v)

30% Polyacrylamide Gel Solution (80:1 Acrylamide:Bis-acrylamide) (Gel shift assays)

29.63%	Acrylamide (w/v)
0.37%	Bis-acrylamide (w/v)

Gel Electrophoresis Sample Buffer

45 mM	Tris-HCl (pH 7.5)
20 mM	EDTA-Na_2
7 M	Urea
0.04%	Xylene Cyanol
0.04%	Bromophenol Blue
10%	Glycerol (v/v)

His-Bind Column Protein Purification Buffers

Binding Buffer
20 mM Tris-HCl (pH 7.9)
5 mM Imidazole
1 M NaCl

Wash Buffer
20 mM Tris-HCl (pH 7.9)
60 mM Imidazole
0.5 M NaCl

Elute Buffer
20 mM Tris-HCl (pH 7.9)
1 M Imidazole
0.5 M NaCl

Column Strip Buffer
20 mM Tris-HCl (pH 7.9)
100 mM $EDTA\text{-}Na_2$
0.5 M NaCl

Column Charging Buffer
50 mM $NiSO_4$

Protein Dialysis Buffers

T7 PK Dialysis Buffer #1
60mM Tris-HCl (pH 7.9)
100mM NH_4Cl

T7 PK Dialysis Buffer #2
60 mM Tris-HCl (pH 7.9)
100 mM NH_4Cl
2 mM $EDTA\text{-}Na_2$
2 mM DTT

RNase III Dialysis Buffer (RNase III, dsRBD and Catalytic domain) #1
60 mM Tris-HCl (pH 7.0)
1 M NaCl

RNase III Dialysis Buffer (RNase III, dsRBD and Catalytic domain) #2
60 mM Tris-HCl (pH 7.0)
1 M NaCl
2 mM $EDTA\text{-}Na_2$
2 mM DTT

SDS-PAGE Buffers

Stacking Gel Buffer (pH 6.8)
- 0.32 M Tris base
- 1% SDS (w/v)
- 10 mM $EDTA\text{-}Na_2$

Resolving Gel Buffer (pH 8.8)
- 0.5 M Tris base
- 0.4% SDS (w/v)
- 2 mM $EDTA\text{-}Na_2$

Electrophoresis Running Buffer
- 25 mM Tris base
- 0.4% SDS
- 0.2 M Glycine

Ammonium Persulfate
- 10% Ammonium Persulfate (w/v)

SDS-PAGE sample loading Buffer (10x)
- 50 mM Tris-HCl (pH 6.8)
- 1% SDS (w/v)
- 1% β-Mercaptoethanol (v/v)
- 10% Glycerol (v/v)
- 20 mM $EDTA\text{-}Na_2$
- 1% Bromophenol Blue (w/v)

30% SDS PAGE Acrylamide Solution
- 29.2% Acrylamide (w/v)
- 0.8% Bis-acrylamide (w/v)

Coomassie Brilliant Blue Staining Solution
- 50% Methanol (v/v)
- 10% Glacial Acetic Acid (v/v)
- 0.05% Coomassie Brilliant Blue R-250 (w/v)

Destaining Buffer
- 5% Methanol (v/v)
- 7% Glacial Acetic Acid (v/v)

2D IEF Gel Buffers

IEF Acrylamide Mix
- 24.3 g Urea
- 5.9 ml 30% Acrylamide (29.2% Acrylamide, 0.8% bis-acrylamide)
- 10.1 ml 10% Triton X-100
- 2.25 ml Ampholines (pH 5-7)
- 0.25 ml Ampholines (pH 3-10)
- 14.4 ml H_2O

Anode Buffer

0.06% Phosphoric acid (v/v)

Cathode Buffer

20 mM NaOH

Lysis Buffer (10 ml)

7 M Urea
2 ml 10% Triton X-100
1% β-Mercaptoethanol (v/v)
0.4 ml Ampholines (pH 5-7)
0.1 ml Ampholines (pH 3-10)

Sample Overlay Buffer (10 ml)

7 M Urea
0.2 ml Ampholines (pH 5-7)
0.05 ml Ampholines (pH 3-10)

SDS/βME Solution (100ml)

0.3 g SDS
5 ml β-Mercaptoethanol
0.144 g Tris-HCl (pH 7.0)
0.265 g Tris base

RESULTS

The expression of the T7 protein kinase in the infected *E. coli* cell induces the phosphorylation of RNase III, and a stimulation in RNase III processing activity (Mayer and Schweiger, 1983; Robertson *et al.*, 1994). Figures 18 and 19 display a representative double (^{35}S, ^{32}P) radiolabeling of T7-infected cell protein. The ^{32}P-protein pattern reveals the occurrence of phosphorylated RNase III, among other phosphorylated species. However, an *in vitro* system is required to determine how *E. coli* RNase III activity is regulated by the T7 protein kinase. The system would include: purified RNase III, T7 PK (Figure 20), and specific RNase III substrates. The pET plasmid "His-tag" system was used to overexpress and purify the proteins used in the *in vitro* system. The RNase III substrates were enzymatically synthesized, purified, and used to measure cleavage reactivity and binding affinity toward phosphorylated and nonphosphorylated RNase III.

A. Overexpression and purification of T7 PK and the T7 PK[G76F] mutant.

The T7 PK is a highly active phosphotransferase, and also undergoes autophosphorylation on serine (E.S. Robertson and A.W. Nicholson, unpublished). Autophosphorylation inactivates phosphotransferase activity (P. Sharma and A.W. Nicholson, unpublished). Although the T7 PK is active as purified from the cell (Figure 21, lane 1), it was suspected that during the overexpression and purification steps, the protein kinase may have undergone autophosphorylation (or phosphorylation by another protein kinase), and therefore exhibit suboptimal activity. To determine if this is the case, the His-T7 PK was treated with purified phage λ protein phosphatase. The "dephosphorylated" T7 PK exhibits a slightly greater electrophoretic mobility compared to untreated T7 PK in a 15% polyacrylamide gel containing SDS (data not shown). The dephosphorylated T7 PK also exhibits greater autophosphorylation activity (Figure 21) (as

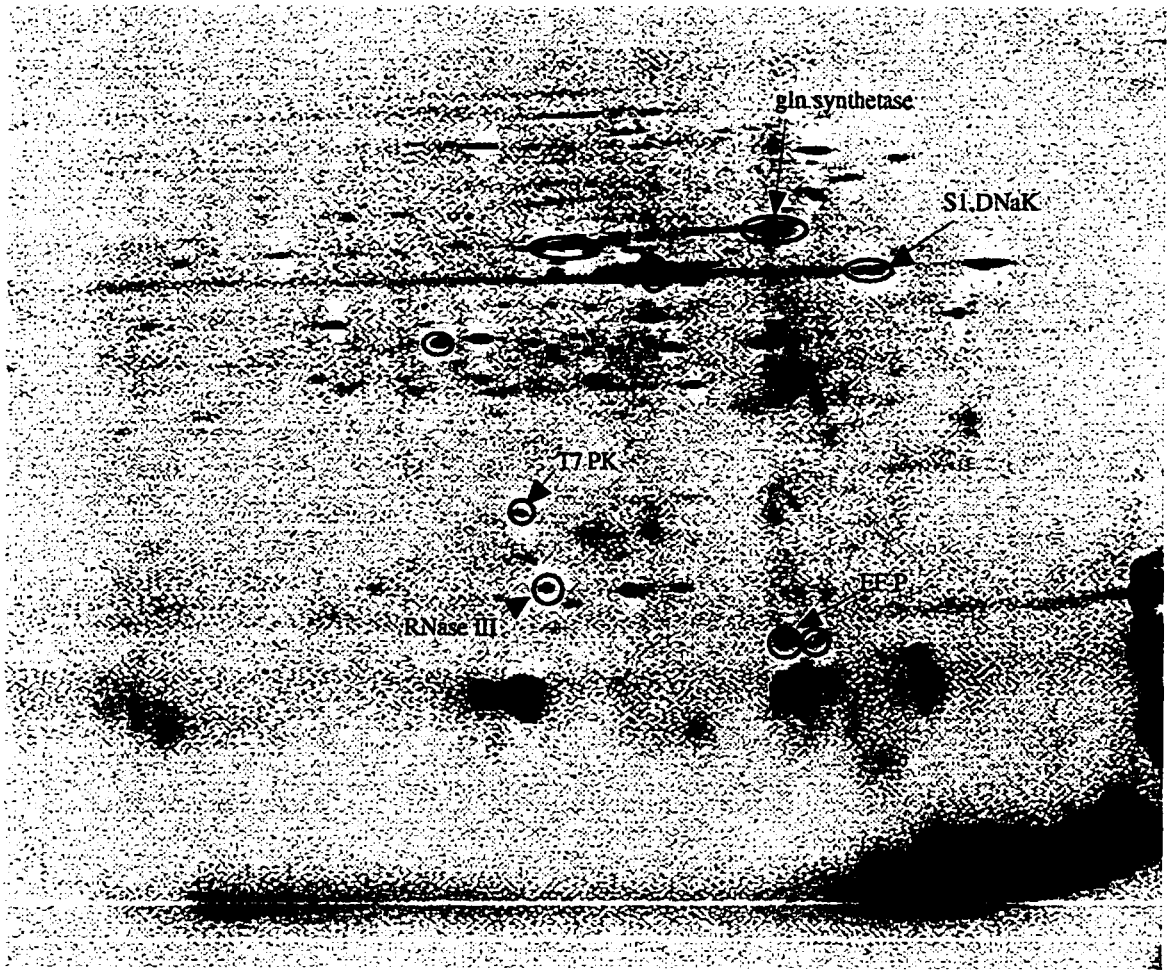


Figure 18. 2-dimensional IEF gel analysis of T7-infected cell protein (^{35}S pattern). An autoradiogram of a 2-dimensional IEF gel of [^{35}S, ^{32}P] double-labeled proteins from T7-infected W3110 *E. coli* (m.o.i.=10). W3110 cells were grown in modified MOPS media, radiolabelled and infected with T7 bacteriophage (see **Materials and Methods**). Cell extracts were prepared and proteins electrophoresed in a first dimension IEF tube gel (pH 3-10). The tube gel was then electrophoresed in a discontinuous SDS-polyacrylamide gel and differential autoradiography performed. Major phosphorylated proteins are circled in red. The corresponding ^{32}P autoradiogram is displayed in Figure 19.

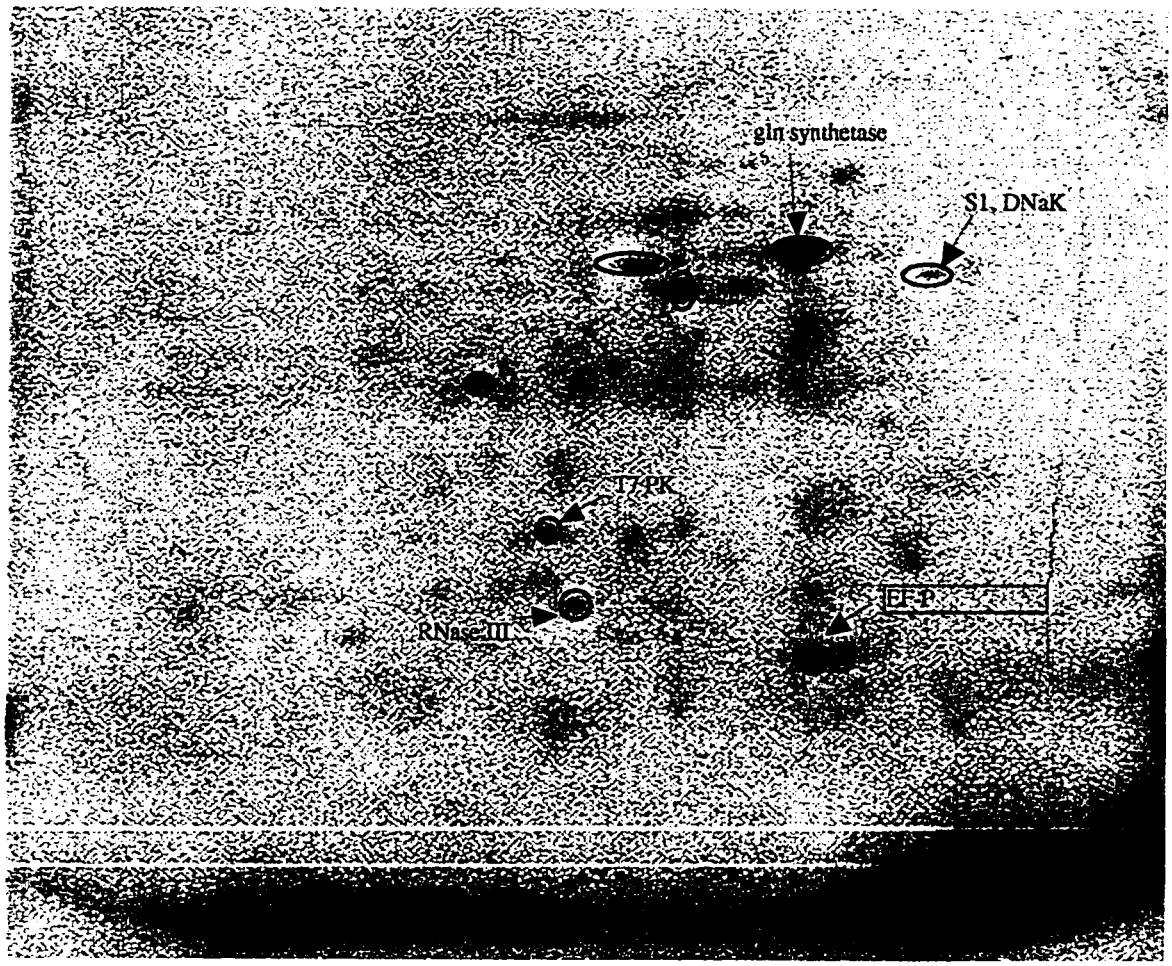


Figure 19. 2-dimensional IEF gel analysis of T7-infected cell protein (^{32}P pattern). Major phosphorylated proteins are circled in red. The corresponding ^{35}S-autoradiogram is displayed in Figure 18

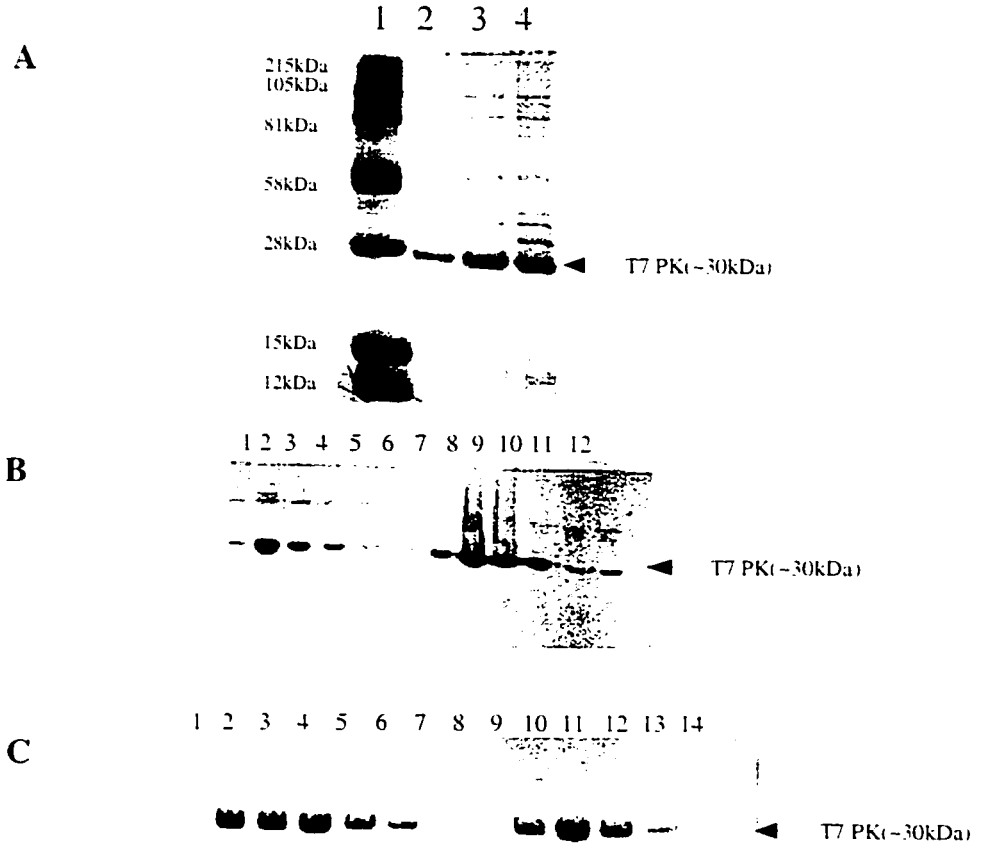


Figure 20. Purification of the T7 PK, and the T7 PK[G76F] mutant. Panel A. Coomassie stained 12% SDS PAGE analysis of T7 PK and T7 PK[G76F] overexpression. Lane 1, prestained protein size markers (sizes are given in kDa). Lane 2, purified T7 PK. Lane 3, proteins from HMS174(DE3) pET-15b(*PK*) cells 3 hours post-treatment with IPTG. Lane 4, proteins from HMS174(DE3) pET-15b(*PK* [G76F]) cells 3 hours post-treatment with IPTG. **Panel B.** Coomassie stained 12% SDS PAGE analysis of T7 PK and T7 PK[G76F] purification. Lanes 1-6, T7 PK eluted fractions 1-6. Lanes 7-12, T7 PK[G76F] eluted fractions 1-6. **Panel C.** Coomassie stained 12% SDS PAGE analysis of T7 PK and T7 PK[G76F] purification. T7 PK and T7PK were dephosphorylated and repurified on fresh His-bind columns (see **Protein overexpression and purification**). Lanes 1-7, T7 PK eluted fractions 1-7. Lanes 8-14, T7 PK[G76F] eluted fractions 1-7.

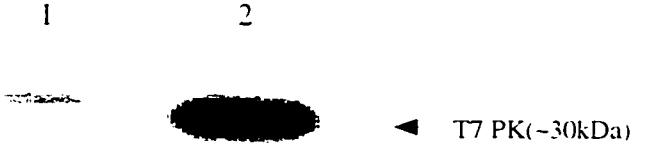


Figure 21. The T7 PK is autophosphorylated and exhibits suboptimal activity as directly purified from *E. coli* cells. The figure displays the relevant portion of an autoradiogram of a 15% SDS PAGE analysis of T7 PK autophosphorylation (as described in **Materials and Methods**). Lane 1. T7 PK autophosphorylation (level of phosphorylation <1% pmols of phosphate/pmols of T7 PK. Lane 2. T7 PK autophosphorylation after treatment of T7 PK with λ protein phosphatase. the level of phosphorylation is ~20% (pmols of phosphate/pmols of T7 PK).

well as phosphotransferase activity, data not shown) as compared to untreated T7 PK.

B. The T7 protein kinase directly phosphorylates RNase III, the RNase III dsRBD, and the RNase III catalytic domain.

RNase III, the RNase III dsRBD, RNase III dsRBD [Serine→Alanine] mutants, RNase III catalytic domain, RNase III[E117K], and RNase III[S195A,S198A] were purified and examined to determine if it they were substrates for T7 PK. All proteins were electrophoretically pure (Figures 22-24) and biochemically active.

The first indication that RNase III is a substrate for T7 PK was inferred from T7 infection experiments (Mayer and Schweiger, 1983; Robertson *et al.*, 1994). To determine if RNase III is a substrate for T7 PK, an *in vitro* protein phosphorylation assay (see **Materials and Methods**) was employed. This assay used purified T7 PK, purified RNase III, and [γ-^{32}P]ATP as the phosphate donor. Figure 25 displays an autoradiogram of an SDS-PAGE analysis of a representative protein phosphorylation assay. Lane 1 shows that the T7 PK undergoes autophosphorylation. The estimated level of phosphorylation is 20% (100 x pmol phosphate/pmol T7 PK). Lane 2 shows that the T7 PK can directly phosphorylate RNase III. The level of phosphorylation of RNase III is ~15% (100 x pmol phosphate/pmol of RNase III). Thus, RNase III is a substrate for T7 PK.

The T7 PK phosphotransfer activity is inhibited by autophosphorylation (Pai *et al.*, 1975b; P. Sharma and A.W. Nicholson, unpublished). Thus, during *in vitro* phosphorylation reactions it is possible that the T7 PK becomes inactive before it is able to phosphorylate all of the available target sites in RNase III. To test this hypothesis, an *in vitro* ^{32}P protein kinase reaction (see **Materials and Methods**) was performed with RNase III as the substrate. At the end of the reaction an additional 1 µg of T7 PK was placed into the reaction tube. The amount of phosphate incorporated into RNase III was unchanged as

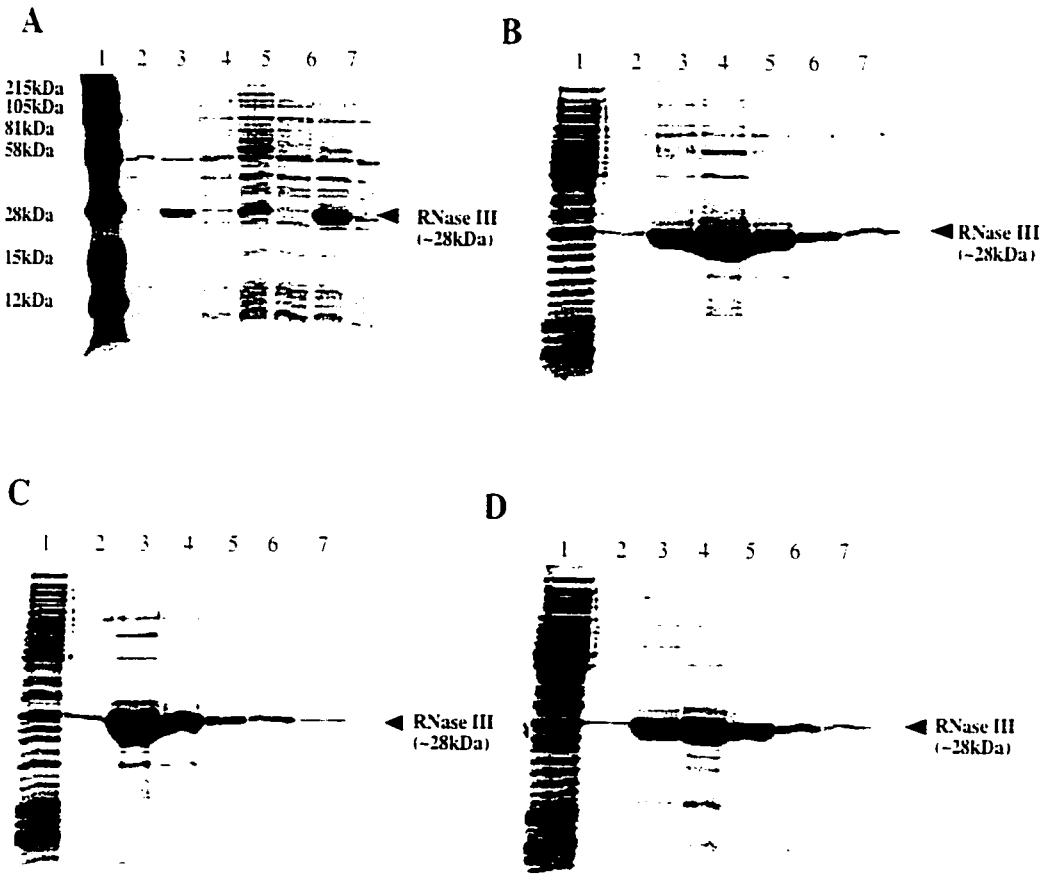


Figure 22. Purification of RNase III, and the RNase III[E117K] and RNase III[S195A,S198A] mutants. Panel A. Coomassie stained 12% SDS PAGE analysis of RNase III overexpression. Lane 1, prestained protein size markers (sizes are given in kDa). Lane 2, proteins from BL21(DE3) pET-15b(*mc*) cells without IPTG treatment. Lane 3, proteins from IPTG-treated BL21(DE3) pET-15b(*mc*) cells. Lane 4, proteins from BL21(DE3) pET-15b(*mc*)[E117K] cells without IPTG treatment. Lane 5, proteins from IPTG-treated BL21(DE3) pET-15b(*mc*)[E117K] cells. Lane 6, proteins from BL21(DE3) pET-15b(*mc*)[S195A,S198A] cells without IPTG treatment. Lane 7, proteins from IPTG-treated BL21(DE3) pET-15b(*mc*)[S195A,S198A] cells. **Panel B.** Coomassie stained 12% SDS PAGE analysis of RNase III purification. **Panel C.** Coomassie stained 12% SDS PAGE analysis of RNase III[E117K] purification. **Panel D.** Coomassie stained 12% SDS PAGE analysis of RNase III[S195A,S198A] purification. For **Panels B-D:** Lane 1, flow-through from His-bind column. Lanes 2-7, eluted fractions 1 through 6.

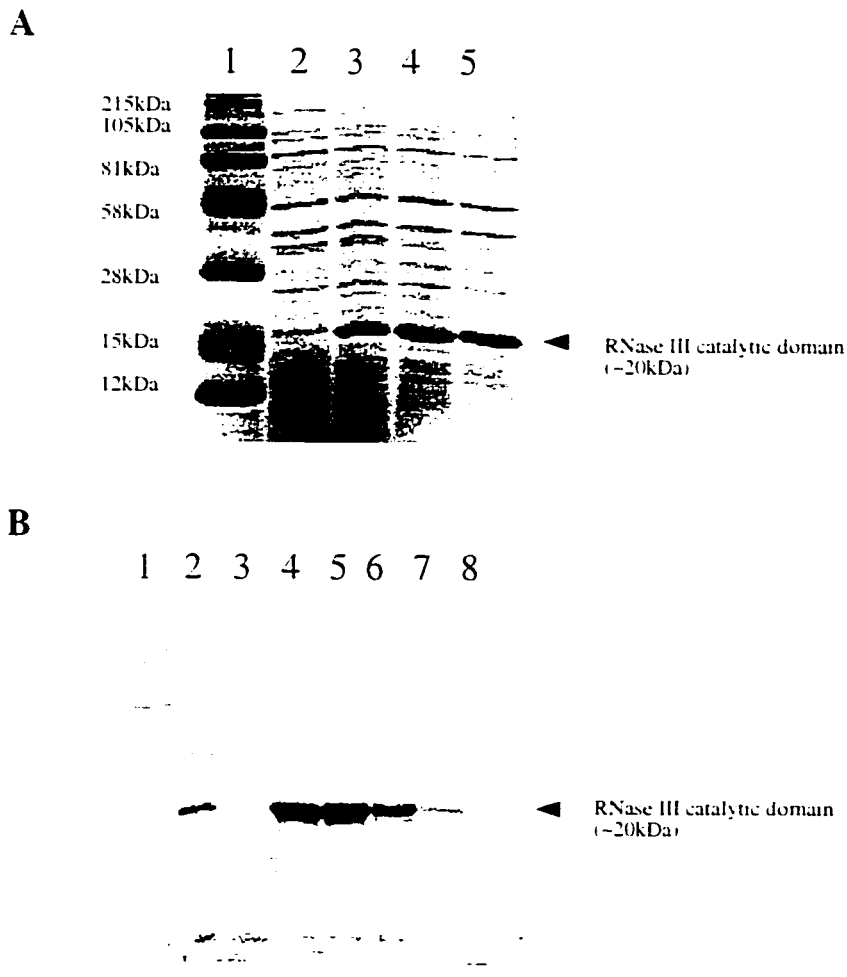


Figure 23. Purification of the RNase III catalytic domain. Panel A: Coomassie stained 12% SDS PAGE analysis of catalytic domain (His-tagged) overexpression. Lane 1. prestained protein size markers (sizes are given in kDa). Lane 2. proteins from BL21(DE3) pET-15b(*catalytic domain*) cells without IPTG treatment. Lane 3. proteins from BL21(DE3) pET-15b(*catalytic domain*) cells 1 hour post-treatment with IPTG. Lane 4. proteins from BL21(DE3) pET-15b(*catalytic domain*) cells 2 hours post-treatment with IPTG.. Lane 5. proteins from BL21(DE3) pET-15b(*catalytic domain*) cells 3 hours post-treatment with IPTG. **Panel B:** Coomassie stained 12% SDS PAGE analysis of catalytic domain purification. Lane 1. uninduced cell culture. Lane 2. IPTG-induced cell culture. Lane 3. flow-through from His-bind column. Lanes 4-8. fractions 1. 2. 3. and 4 from column.

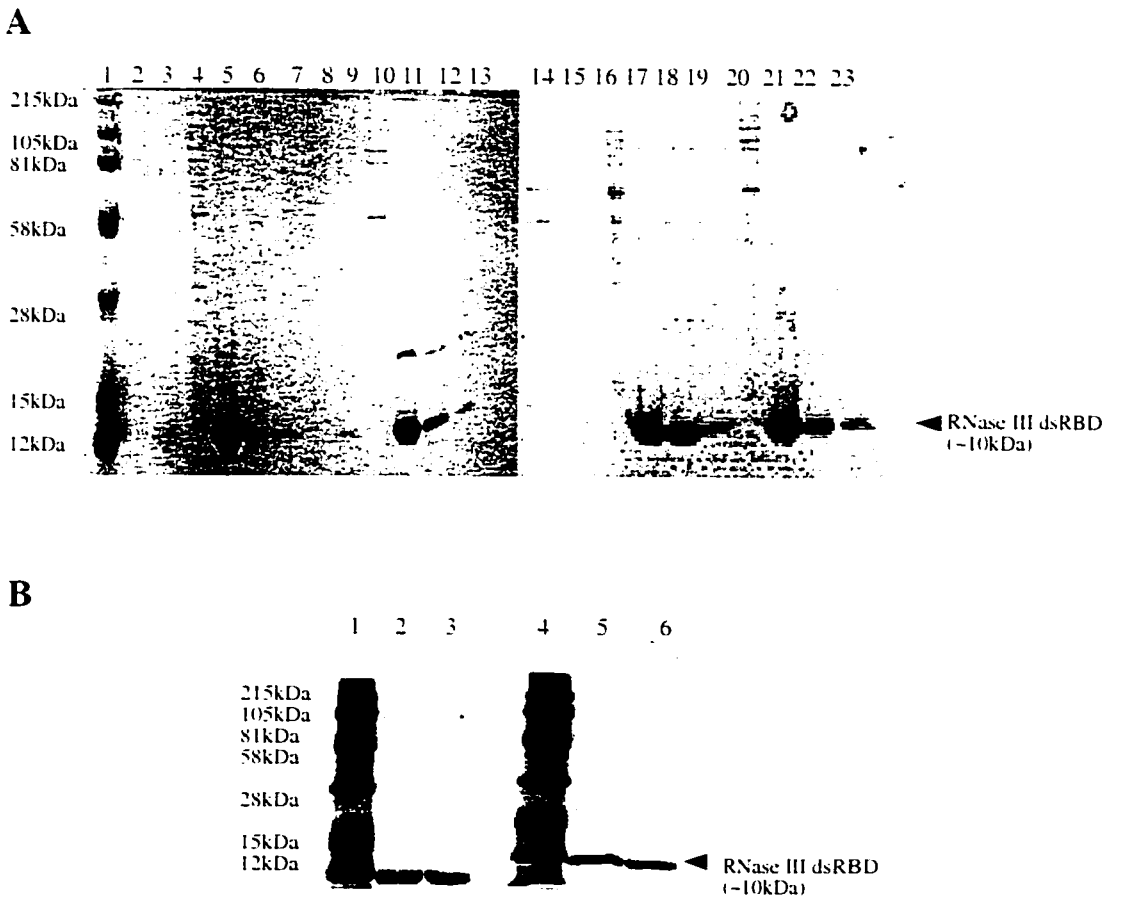


Figure 24. Purification of RNase III dsRBD, dsRBD[S195A], dsRBD [S198A], and dsRBD[S206A,S207A]. **Panel A:** Coomassie stained 12% SDS PAGE analysis of dsRBD purification. Lane 1, prestained protein size markers (sizes are given in kDa). Lanes 2-7, RNase III dsRBD purification. Lane 2, proteins from BL21(DE3) pET-15b(*dsRBD*) cells without IPTG treatment. Lane 3, proteins from IPTG-treated BL21(DE3) pET-15b(*dsRBD*) cells. Lane 4, flow through from column. Lanes 5-7, elute fractions 1, 2, and 3 from column. Lanes 8-13, RNase III dsRBD[S195A] purification. Lane 8, proteins from BL21(DE3) pET-15b(*dsRBD*)[S195A] cells without IPTG treatment. Lane 9, proteins from IPTG-treated BL21(DE3) pET-15b(*dsRBD*)[S195A] cells. Lane 10, flow through from column. Lanes 11-13, elute fractions 1, 2, and 3 from column. Lanes 14-19, RNase III dsRBD[S198A] purification. Lane 14, proteins from BL21(DE3) pET-15b(*dsRBD*)[S198A] cells without IPTG treatment. Lane 15, proteins from IPTG-treated BL21(DE3) pET-15b(*dsRBD*)[S198A] cells Lane 16, flow through from column. Lanes 17-19, eluted fractions 1, 2, and 3. Lanes 20-23, RNase III dsRBD[S206A,S207A] purification. Lane 20, flow through from column. Lanes 21-23, RNase III dsRBD(S206A,S207A) eluted fractions 1, 2, and 3 from column. **Panel B:** Coomassie stained SDS-PAGE (12%) analysis of dsRBD purification. Individual dsRBDs were repurified on fresh His-bind columns and the elute 2 fraction electrophoresed in a 12% SDS-polyacrylamide gel. Lanes 1 and 4, prestained protein size markers (sizes are given in kDa). Lane 2, RNase III dsRBD. Lane 3, RNase III dsRBD[S195A]. Lane 5, RNase III dsRBD[S198A]. Lane 6, RNase III dsRBD[S206A,S207A].

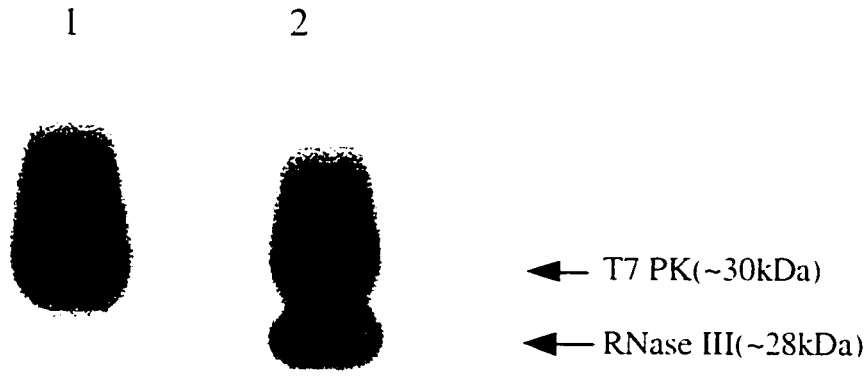


Figure 25. ***In vitro*** **phosphorylation of RNase III by T7 PK and autophosphorylation of T7 PK.** The figure displays the relevant portion of an autoradiogram of an SDS-containing 15% polyacrylamide gel analysis of phosphorylation of RNase III by T7 PK (see **Materials and Methods**). The T7 PK and substrate were present in equimolar amounts (33 pmols each) in the phosphorylation reactions. Protein phosphorylation reaction volumes were adjusted to maintain a final glycerol concentration <10%. Reactions were initiated with the addition of Mg^{+2}, and stopped by adding SDS sample buffer containing EDTA. The positions of T7 PK (~30 kDa) and RNase III (~28 kDa) are indicated. Lane 1. T7 PK. Lane 2. T7 PK + RNase III.

compared to the standard protein kinase reaction (data not shown). It is concluded therefore, that while the calculated minimal level of phosphorylation is ~15%, the phosphorylation reaction reaches a plateau of ≥15%.

It was found that a glycerol concentration of >10% inhibits the transfer of phosphate to substrate, as well as inhibiting autophosphorylation (data not shown). It was also determined that the point at which Mg^{+2}/ATP is added to the reaction is essential for efficient transfer of phosphate to substrate (Figure 26). Optimal conditions consisted of combining dephosphorylated T7 PK and substrate in reaction buffer, prior to addition of Mg^{+2}/ATP.

The RNase III double-stranded-RNA-binding domain (dsRBD) contains four serines (Ser195, Ser198, Ser206, and Ser207 see Figure 2), one or more of which could be phosphorylated by the T7 PK. The RNase III dsRBD, and a set of serine→alanine dsRBD mutants were purified and tested for their ability to be phosphorylated *in vitro* by T& PK. The dsRBD mutants were: dsRBD[S195A], dsRBD[S198A], dsRBD[S207A], and dsRBD[S206A,S207A]. An autoradiogram of an SDS-containing, 12% polyacrylamide gel is displayed in Figure 27. The dsRBD, dsRBD[S207A], and dsRBD[S206A,S207A] proteins are phosphorylated by T7 PK (Figure 27, Lanes 1, 4, and 5). These results rule out serine 206 and serine 207 as targets. However, the dsRBD[S195A] and dsRBD[S198A] proteins are not phosphorylated, indicating that serine 195 and/or serine 198 is phosphorylated by the T7 PK. In addition, these results show that mutation of one of the two serines negatively influences the ability of the other to be phosphorylated. Thus, if serine 195 is the phosphorylation target, then mutation of serine 198 to alanine prevents phosphorylation of serine 195 (Figure 27, Lane 2). Conversely, if serine 198 is the phosphorylated residue, then mutation of serine 195 to alanine prevents phosphorylation of the serine 198 (Figure 27, Lane 3). If both serine 195 and 198 are phosphorylated, then mutation of either residue is sufficient to prevent phosphorylation of both (Figure 27, Lanes 2 and 3). It may be argued that mutation of the putative non-target

residue causes a protein conformational change, which would prevent phosphorylation of the other serines in the dsRBD (i.e. serine 206 and/or 207). However, the sustained substrate binding and cleavage activity of the RNase III[S195A,S198A] double mutant (see below), and the ability of the dsRBD[S207A] and dsRBD [S206A,S207A] mutants to be phosphorylated (Figure 27, Lane 4 and 5), suggest that such a protein conformational change, if it occurs, is not severe. An alternative explanation is that if either serine 195 or serine 198 are phosphorylated, then the other (non-target) serine acts as a recognition determinant for T7 PK.

The availability of purified RNase III catalytic domain allowed an examination of potential phosphorylation sites in this portion of the RNase III polypeptide. Figure 28 is an autoradiogram of an SDS-containing, 15% polyacrylamide gel analysis of T7 PK phosphorylation of: RNase III dsRBD, RNase III catalytic domain, RNase III, RNase III[E117K] mutant, and RNase III[S195A,S198A] mutant (as described in **Materials and Methods**). All substrates are phosphorylated by T7 PK. Of particular interest is that the catalytic domain of RNase III is phosphorylated by T7 PK. Also, RNase III [S195A,S198A] is phosphorylated, in contrast to the inability of the dsRBD[S195A] and dsRBD[S198A] polypeptides to be phosphorylated (Figure 27, Lanes 2 and 3). Taken together, these experiments demonstrate: **1)** *E. coli* RNase III is a substrate for the T7 protein kinase and **2)**, the targets of phosphorylation are at least one phosphoserine in the dsRBD (serine 195 and/or serine 198), and one or more serines in the catalytic domain.

C. Phosphorylation of RNase III enhances the cleavage of T7 processing substrates

Although it is now demonstrated that RNase III is phosphorylated by the T7 PK, it is not known whether this covalent modification is responsible for modifying the enzymatic activity of RNase III. Furthermore, it is not known whether an alteration in enzymatic

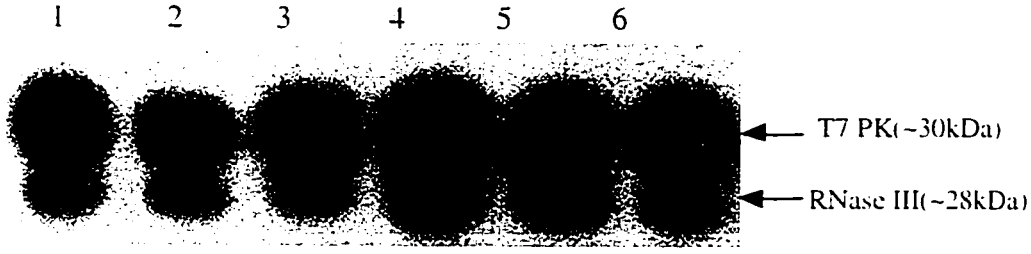


Figure 26. ***In vitro*** **phosphorylation of RNase III, RNase III[E117K] mutant, and RNase III[S195A,S198A] mutant.** The figure displays the relevant portion of an autoradiogram of an SDS-containing 12% polyacrylamide gel analysis of phosphorylation of RNase III by T7 PK (see **Materials and Methods**). A 10x mixture of Mg^{+2} (150mM) + 10 µCi of [γ^{32}P] ATP was made just prior to each protein kinase reaction. The addition of components were in the following order: 1) 10x PK assay buffer; 2) H_2O; 3) protein substrate; 4) T7 PK (incubation at 30°C for 15 minutes); and 5). 10x Mg^{+2}/ATP. Addition of ATP at any other point in the reaction results in a less efficient reaction The positions of His-T7 PK (~30 kDa) and RNase III (~28 kDa) are indicated. Lanes 1 and 4. RNase III. Lanes 2 and 5. RNase III[E117K]. Lanes 3 and 6. RNase III[S195A,S198A]. Lanes 1-3 are protein kinase reactions in which [γ-^{32}P]ATP is added before the addition of T7 PK. Lanes 4-6 are protein kinase reactions in which the [γ-^{32}P] ATP is added following incubation of T7 PK and RNase III, as described in text. The level of phosphorylation of RNase III in lanes 1-3 is ~1.8% (pmols of phosphate/pmols of RNase III). The level of phosphorylation of RNase III in lanes 4-6 is ~15% (pmols of phosphate/pmols of RNase III).

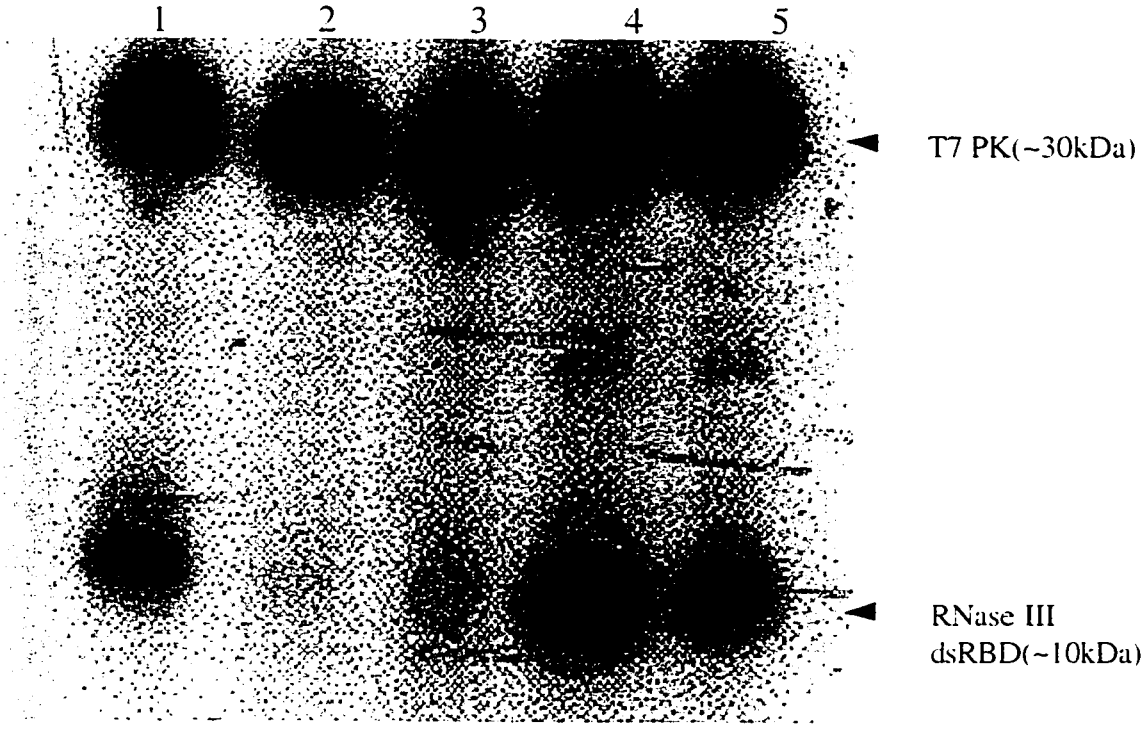


Figure 27. *In vitro* phosphorylation of RNase III dsRBD and dsRBD[serine→alanine] mutants. The figure displays the relevant portion of an autoradiogram of an SDS-containing 15% polyacrylamide gel analysis of phosphorylation of dsRBD. and dsRBD[serine→alanine] mutants by T7 PK (see **Materials and Methods**). The positions of T7 PK and dsRBD are indicated. The positions of T7 PK and dsRBD are indicated. Lane 1. T7 PK+dsRBD. Lane 2. T7 PK+dsRBD[S195A]. Lane 3. T7 PK+dsRBD[S198A]. Lane 4. T7 PK+dsRBD[S207A]. Lane 5. T7 PK+ dsRBD[S206A.S207A].

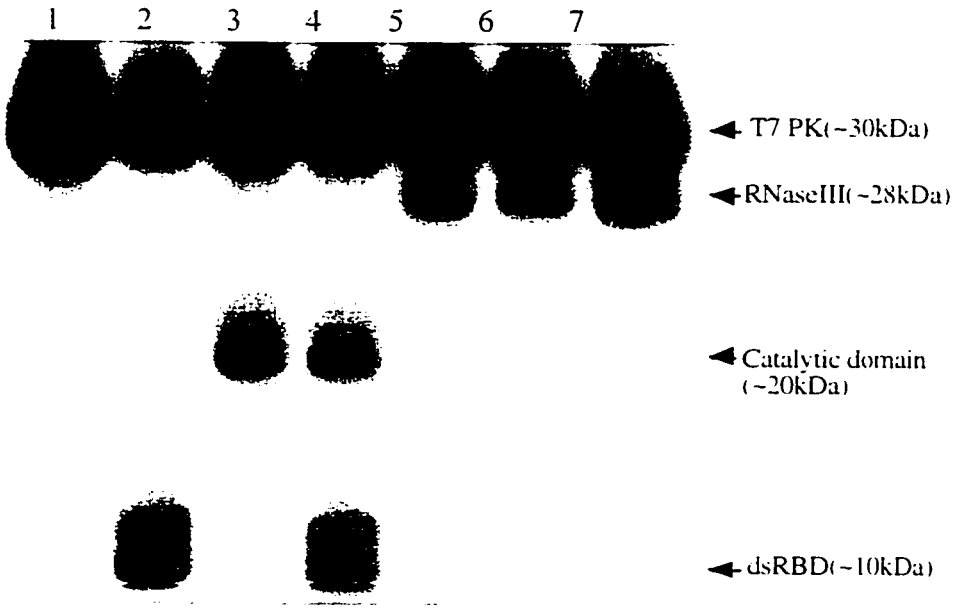


Figure 28. *In vitro* phosphorylation of RNase III, RNase III catalytic domain, and RNase III dsRBD. The figure displays a ^{32}P autoradiogram of a 12% SDS PAGE analysis of T7 PK phosphorylation of protein substrates as described in **Materials and Methods**. The positions of His-T7 PK, His-RNase III, His-catalytic domain, and His-dsRBD are indicated. Lane 1, T7 PK autophosphorylation. Lane 2, T7 PK+dsRBD. Lane 3, T7 PK+catalytic domain. Lane 4, T7 PK+dsRBD+catalytic domain. Lane 5, T7 PK+RNase III. Lane 6, T7 PK+RNase III[E117K]. Lane 7, T7 PK+RNase III[S195A,S198A]. The dsRBD bands are slightly diffuse, because the dsRBD electrophoreses close to the ion front in a 12% polyacrylamide gel containing SDS.

activity (if it occurs) extends to all RNase III substrates. Four RNase III processing substrates were chosen for this analysis, which are representative of the known classes of RNase III processing substrates (T7 early, T7 late, cellular, and model dsRNA). They include: T7 R1.1 RNA, T7 R1.1[WC] RNA, T7 R18.5 RNA, and *mcO* RNA.

The bacteriophage T7 R1.1 processing signal is located in the early region of the T7 genome between genes 1 and 1.1 (see Figure 12). The secondary structure of the T7 R1.1 RNA is a hairpin stem-loop, containing an asymmetric internal loop (Figures 10 and 29). RNase III cleavage occurs at a single site within the internal loop (Dunn and Studier, 1983). The T7 R1.1 RNA used in this study is 60 nts long, and RNase III cleavage occurs 3' of nt U_{47}, creating a 47 nt 5'-end fragment and a 13 nt 3'-end fragment.

T7 R1.1[WC] RNA provides a model dsRNA substrate for RNase III. The T7 R1.1[WC] RNA is a variant of the T7 R1.1 RNA substrate, in which the R1.1 internal loop is altered to produce a fully double-stranded form. The T7 R1.1[WC] RNA is cleaved by RNase III at two sites: one being the canonical site for T7 R1.1 (3' of nt U_{47}), and a second site (3' of nt U_{20}) (Figure 10). Cleavage creates three products: a 20 nt 5'-end fragment, a 34 nt upper-stem fragment, and a 13 nt 3'-end fragment.

The bacteriophage T7 R18.5 processing signal is located in the late region of the T7 genetic map, between genes 18 and 18.5 (see Figure 14). The T7 R18.5 secondary structure is similar to the R1.1 RNA, and includes a hairpin stem-loop with an asymmetric internal loop (Figures 10 and 33). RNase III cleavage occurs on the 3' side of nt U_{58}, creating a 58 nt 5'-end fragment and a 12 nt 3'-end fragment. The T7 R18.5 RNA used in this study is 70 nt.

The *E. coli mcO* RNA represents the 5' RNA leader sequence of the *rnc* operon. The RNase III cleavage sites of the *mcO* leader sequence occur in a double-stranded segment, and are 3' of nt G_{40} and 3' of nt G_{122} (Figures 6 and 35). The *mcO* substrate is 205 nt in length.

A time course for RNase III cleavage was performed to determine (I) the *in vitro* reactivity of each of these processing signals, and (ii) whether phosphorylation of RNase III affects the cleavage rates. All four substrates were enzymatically synthesized as run-off transcripts using synthetic deoxyoligonucleotide templates, and were gel-purified (see **Materials and Methods**). In these experiments, RNase III was pretreated in one of three ways: **1)** "PK-RNase III": RNase III was phosphorylated *in vitro* with T7 PK and ATP, **2)** "G76F-RNase III": RNase III was mock phosphorylated *in vitro* with T7 PK[G76F] and ATP, and **3)**, "RNase III": RNase III was incubated *in vitro* in T7 PK assay buffer and ATP (see **Materials and Methods**). Since the phosphorylation reaction contains 15 mM Mg^{+2}, the carryover of Mg^{+2} from the phosphorylation reaction was sufficient to support cleavage. Therefore, to obtain reproducible kinetic data, substrate cleavage reactions were prepared and the reactions initiated with the addition of RNase III. Moreover, to ensure that initial cleavage rates would be sensitive to changes in K_M or k_{cat} , the concentration of RNase III was ~10 nM (dimer) and the final concentration of RNA was ~50 nM. Both of these concentrations were below the K_M for RNase III cleavage of these substrates (0.2-1 μM). Reactions were stopped with excess EDTA, and aliquots electrophoresed in 15% polyacrylamide gels containing 7 M urea (For *mcO* RNA, the reactions were electrophoresed in a 10% polyacrylamide gel containing 7 M urea, in order to resolve the relatively large RNA fragments).

For each reaction time course, an autoradiogram of a gel was generated, and radioanalytic imaging used to quantitate the kinetics (Figures 29, 31, 33, and 35). Results from the radioanalytic analysis of each time course study are displayed graphically with each autoradiogram (Figures 30, 32, 34, and 36). A comparison of initial velocity (V_i) for each time course experiment was calculated and the results are listed in Table 5.

Comparison of the cleavage rates using "PK-RNase III" and "G76F-RNase III" indicates that the rate of cleavage of T7 R1.1 RNA and T7 R1.1[WC] RNA are increased

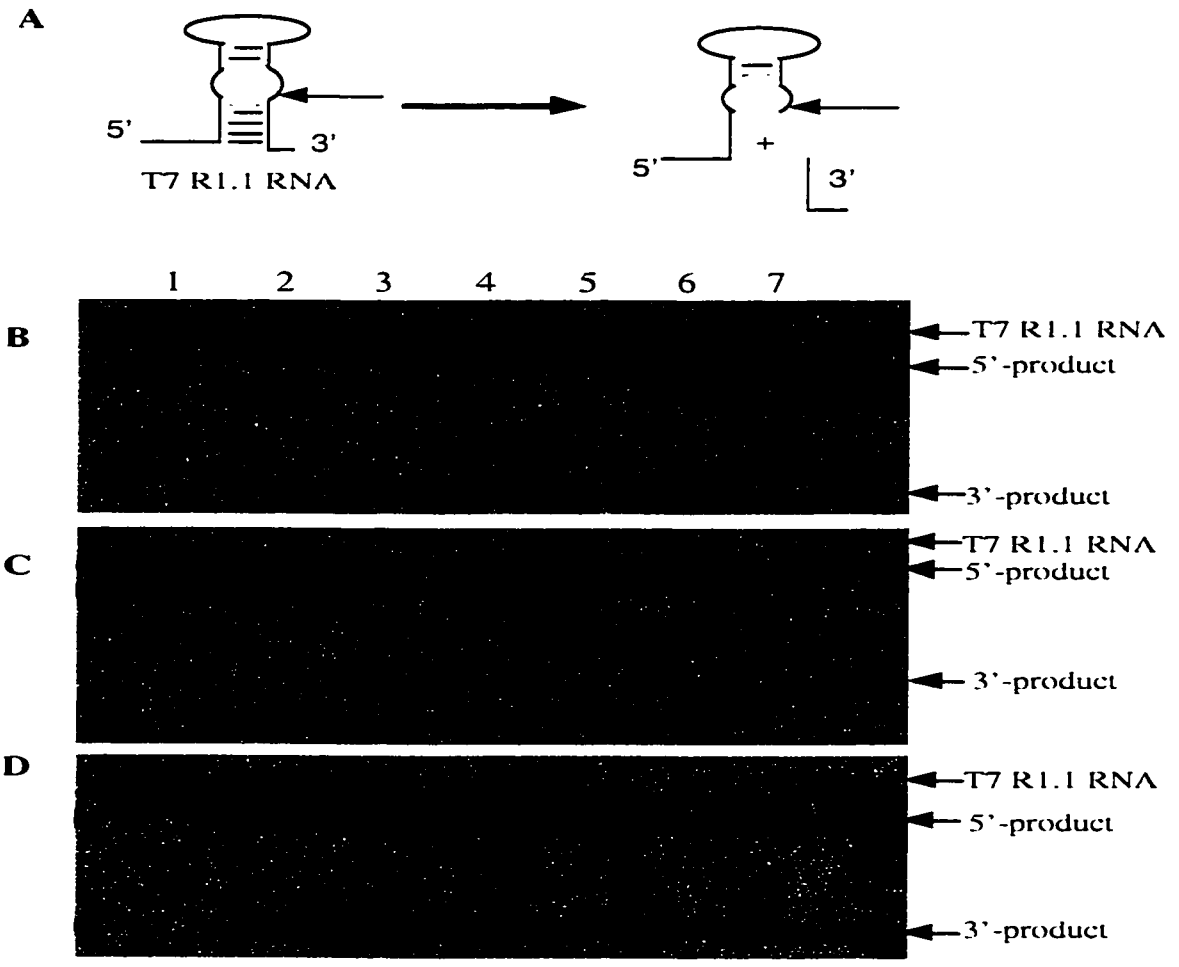


Figure 29. Cleavage of T7 R1.1 RNA is enhanced by RNase III phosphorylation. **Panel A:** Schematic of the cleavage pattern of T7 R1.1 RNA. The cleavage site is indicated with a red arrow. Internally ^{32}P-labeled T7 R1.1 RNA (12,000 dpm; 4 pmol) was incubated with RNase III (10nM; 0.2 pmol) at 37^{0}C in cleavage assay buffer for varying times (Lane 1, 0 minute; Lane 2, 1 minute; Lane 3, 2 minutes; Lane 4, 3 minutes; Lane 5, 4 minutes; Lane 6, 5 minutes; and Lane 7, 10 minutes). Reactions were stopped by adding an equal volume of gel electrophoresis sample buffer, then electrophoresed in a 15% polyacrylamide gel (7M urea). Autoradiography was performed, as well as quantitation by radioanalytic imaging. Position of uncleaved T7 R1.1, 5' and 3'-products are indicated with arrows. **Panel B:** Reaction using mock treated RNase III. **Panel C:** Reaction using T7 PK[G76F] treated RNase III. **Panel D**: Reaction with T7 PK-treated RNase III.

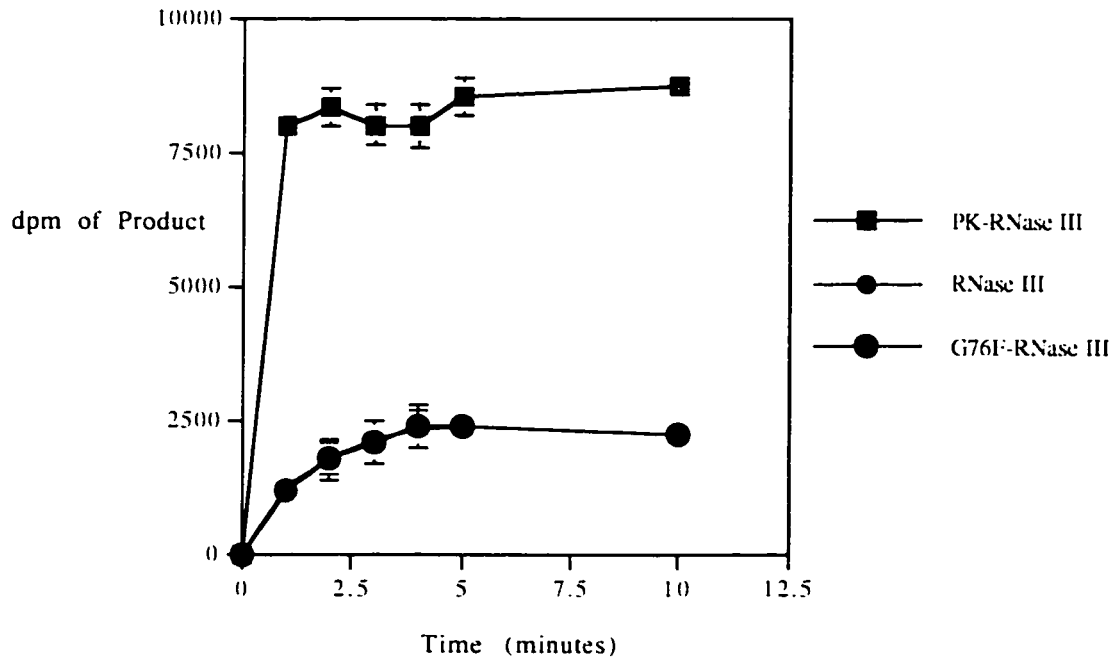


Figure 30. Quantitation of T7 R1.1 RNA cleavage stimulation by phosphorylation of RNase III. Kinetic analysis of T7 R1.1 RNA cleavage. Product amounts were determined by radioanalytic imaging. Black line (PK-RNase III) represents T7 PK-treated RNase III {V_i= 2.64±0.05 pmol min^{-1}}. Red line (RNase III) represents mock-treated RNase III {V_i= 0.41±0.06 pmol min^{-1}}. Green line (G76F-RNase III) represents T7 PK[G76F]-treated RNase III {V_i= 0.39±0.08 pmol min^{-1}}.

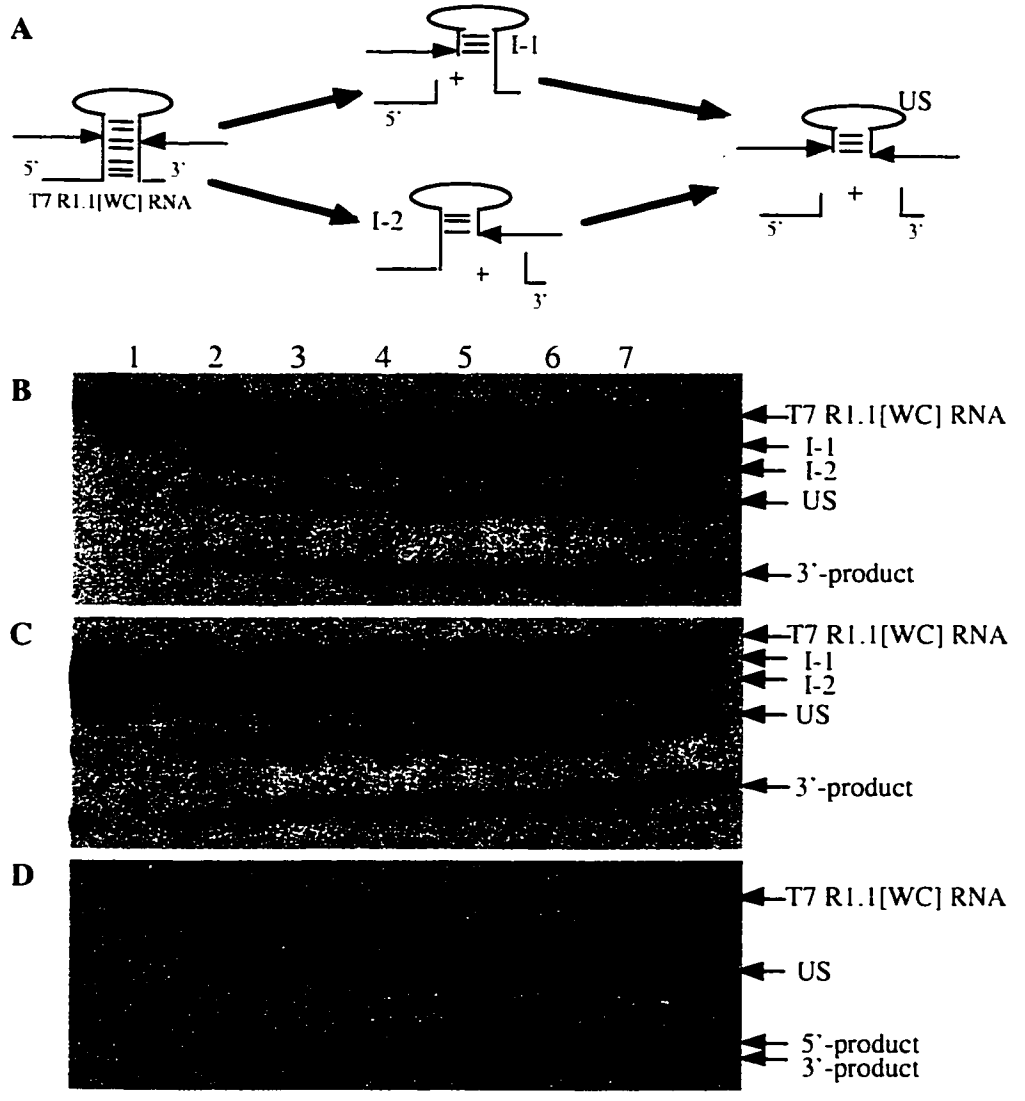


Figure 31. Cleavage of T7 R1.1[WC] RNA is enhanced by RNase III phosphorylation. Panel A: Schematic of the cleavage pattern of T7 R1.1[WC] RNA. Cleavage sites are indicated by the red arrows. Internally ^{32}P-labeled T7 R1.1[WC] RNA (12,000 dpm; 4 pmol) was incubated with RNase III (10nM; 0.2 pmol) at 37°C in cleavage assay buffer for varying times (Lane 1, 0 minute; Lane 2, 1 minute; Lane 3, 2 minutes; Lane 4, 3 minutes; Lane 5, 4 minutes; Lane 6, 5 minutes; and Lane 7, 10 minutes). Reactions were stopped by adding an equal volume of gel electrophoresis buffer then electrophoresed in a 15% polyacrylamide gel (7M urea). Autoradiography was performed using x-ray film and intensifying screens. Position of uncleaved T7 R1.1[WC] RNA, upper-stem (US), 5' and 3'-products are indicated with arrows. Also indicated are the products of single-site cleavage (I-1 and I-2). **Panel B:** mock-treated RNase III. **Panel C:** T7 PK[G76F]-treated RNase III. **Panel D:** T7 PK-treated RNase III.

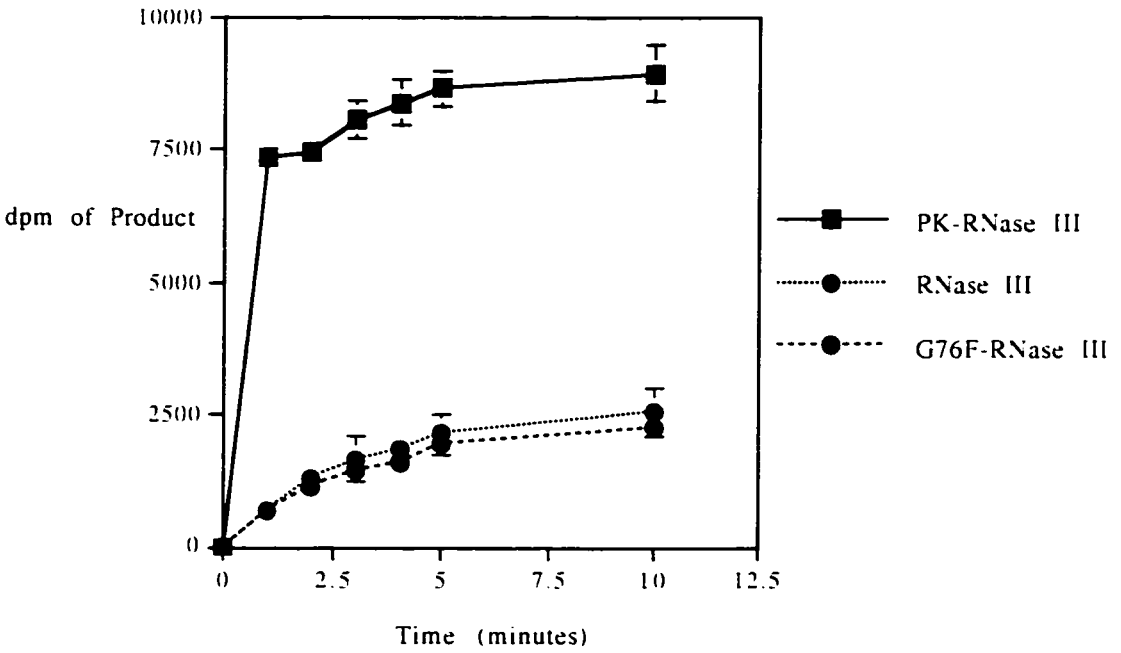


Figure 32. Quantitation of T7 R1.1[WC] RNA cleavage stimulation by phosphorylation of RNase III. Kinetic analysis of T7 R1.1 RNA cleavage. Product amounts were determined by radioanalytic imaging. Black line (PK-RNase III) represents T7 PK-treated RNase III {V_i= 2.42±0.08 pmol min^{-1}}. Red line (RNase III) represents mock-treated RNase III {V_i= 0.22±0.04 pmol min^{-1}}. Green line (G76F-RNase III) represents T7 PK[G76F]-treated RNase III {V_i= 0.22±0.09 pmol min^{-1}}.

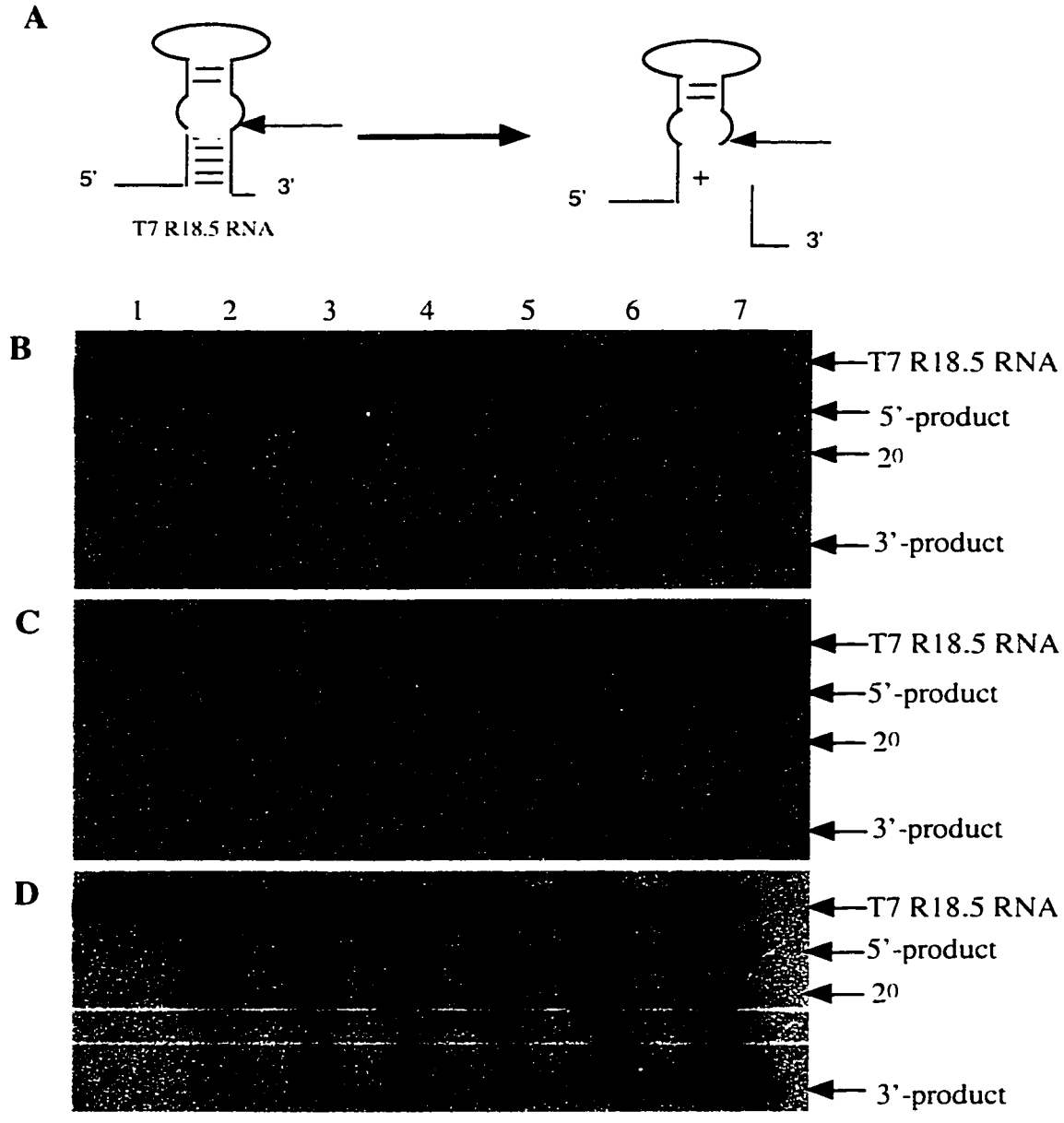


Figure 33. Phosphorylation of RNase III stimulates cleavage of T7 R18.5 RNA. **Panel A:** Schematic of cleavage pattern of T7 R1.1[WC] RNA. The cleavage site is indicated by the red arrow. Internally ^{32}P-labeled T7 R18.5 RNA (12,000 cpm; 4 pmol) was incubated with RNase III (10nM; 0.2 pmol) at 37^{0}C in cleavage assay buffer for varying times (Lane 1, 0 minute; Lane 2, 1 minute; Lane 3, 2 minutes; Lane 4, 3 minutes; Lane 5, 4 minutes; Lane 6, 5 minutes; and Lane 7, 10 minutes). Reactions were stopped by adding an equal volume of gel electrophoresis buffer and electrophoresed in a 15% polyacrylamide gel (7M urea). Autoradiography was performed using x-ray film and intensifying screens. Position of uncleaved T7 R1.1, 5'-end and 3'-end products are indicated with arrows. Also indicated is a 2^{0} cleavage site which has not been mapped. **Panel B:** mock-treated RNase III. **Panel C:** T7 PK[G76F]-treated RNase III. **Panel D:** T7 PK-treated RNase III.

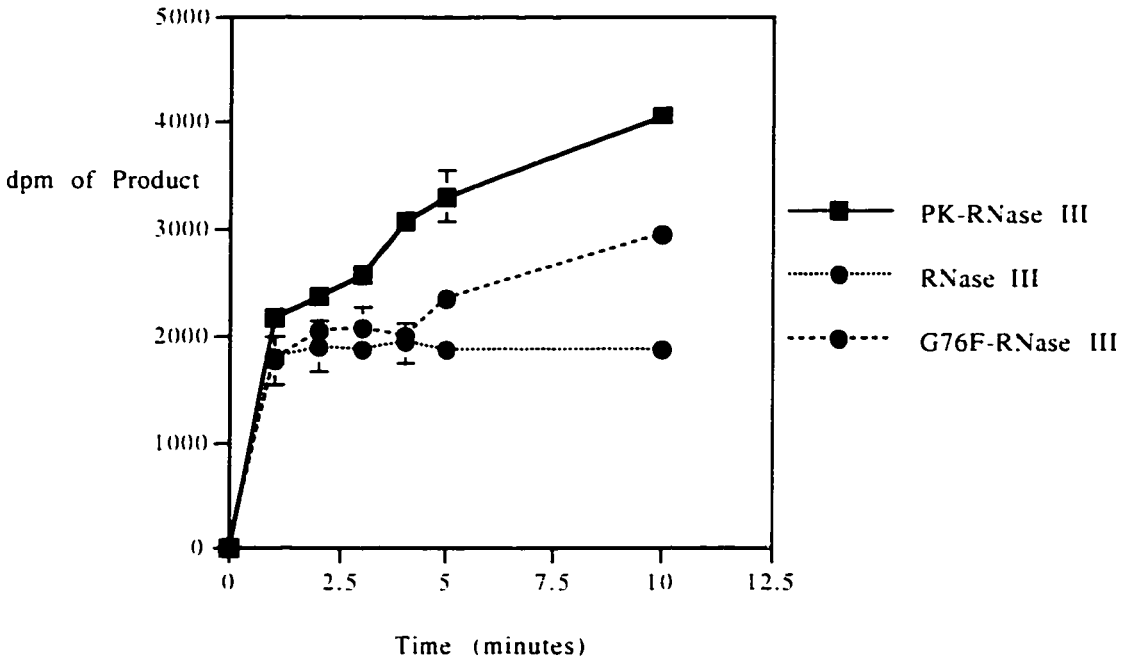


Figure 34. Quantitation of T7 R18.5 RNA cleavage stimulation by phosphorylation of RNase III. Kinetic analysis of T7 R18.5 RNA cleavage. Product amounts were determined by radioanalytic imaging. Black line (PK-RNase III) represents T7 PK-treated RNase III {V_i= 0.72±0.02 pmol min^{-1}}. Red line (RNase III) represents mock-treated RNase III {V_i= 0.59±0.01 pmol min^{-1}}. Green line (G76F-RNase III) represents T7 PK[G76F]-treated RNase III {V_i= 0.58±0.08 pmol min^{-1}}.

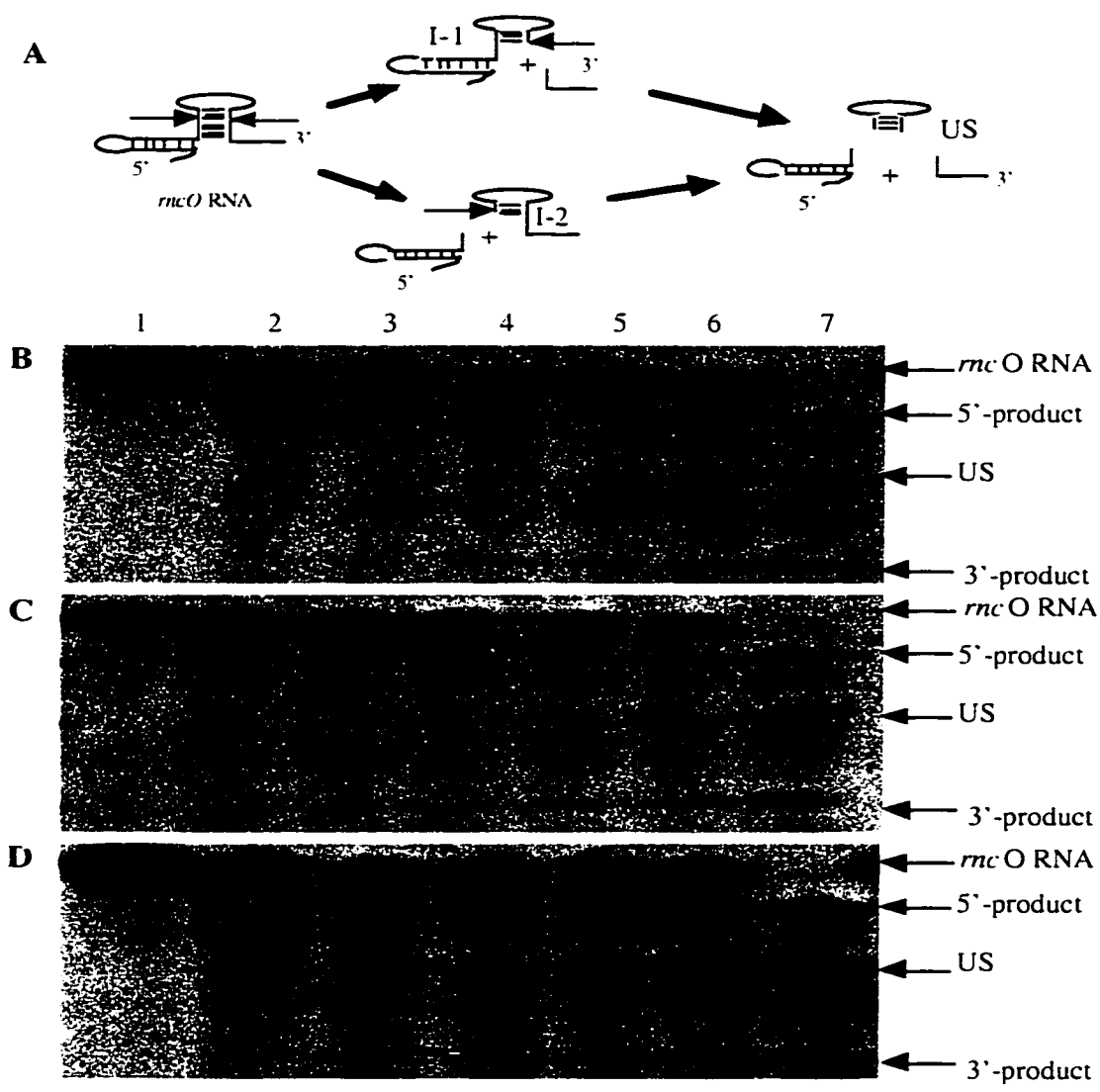


Figure 35. Phosphorylation of RNase III causes moderate stimulation of cleavage of the cellular processing substrate *rncO* RNA. Panel A: Schematic of the cleavage pattern of *rncO* leader RNA (Matsunga *et al.*, 1997). Cleavage sites are indicated by the red arrows.. Internally ^{32}P-labeled *rncO* RNA (36,000 dpm; 4 pmol) was incubated with RNase III (10nM; 0.2 pmol) at 37^{0}C in cleavage assay buffer for varying times (Lane 1, 0 minute; Lane 2, 1 minute; Lane 3, 2 minutes; Lane 4, 3 minutes; Lane 5, 4 minutes; Lane 6, 5 minutes; and Lane 7, 10 minutes). Reactions were stopped by adding an equal volume of gel electrophoresis sample buffer, then electrophoresed in a 10% polyacrylamide gels (7M urea). Autoradiography was performed using x-ray film and intensifying screens. Position of uncleaved *rncO* RNA, upper-stem (US), 5' and 3' products are indicated. The other bands represent minor amounts of cleavage at 2^{0} sites. **Panel B:** mock-treated RNase III. **Panel C:** T7 PK[G76F]-treated RNase III. **Panel D:** T7 PK-treated RNase III.

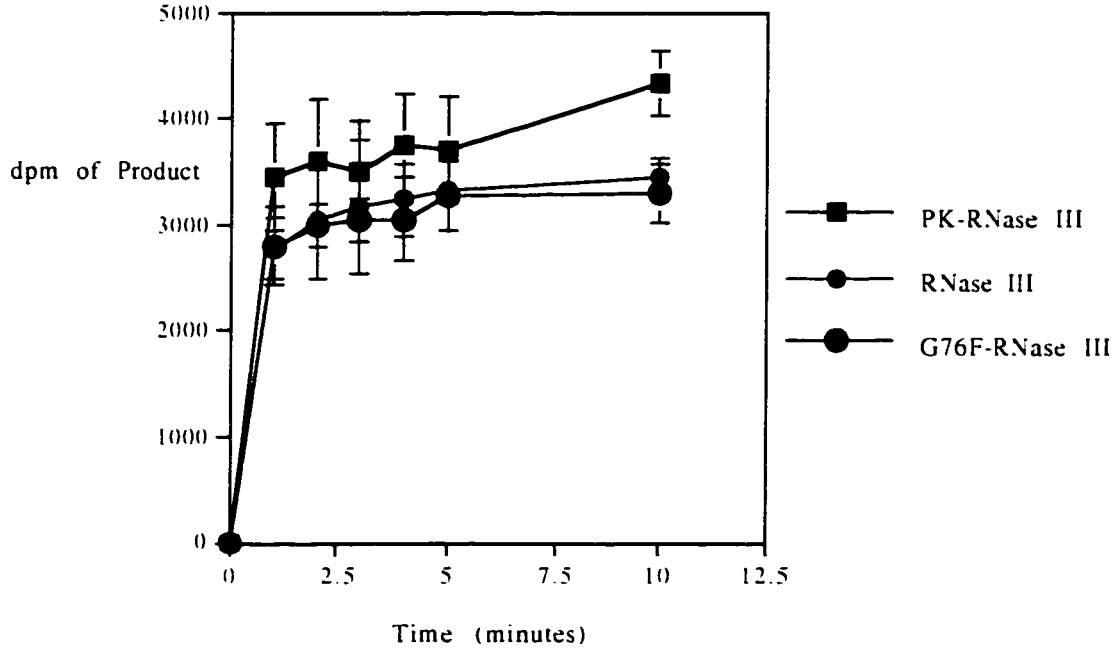


Figure 36. Quantitation of *rncO* leader RNA cleavage stimulation by phosphorylation of RNase III. Kinetic analysis of T7 R18.5 RNA cleavage. Product amounts were determined by radioanalytic imaging. Black line (PK-RNase III) represents T7 PK-treated RNase III {V_i= 1.14±0.17 pmol min^{-1}}. Red line (RNase III) represents mock-treated RNase III {V_i= 0.91±0.09 pmol min^{-1}}. Green line (G76F-RNase III) represents T7 PK[G76F]-treated RNase III {V_i= 0.92±0.12 pmol min^{-1}}.

6.5-fold and 11-fold, respectively with phosphorylation (Figures 30 and 32). The processing of T7 R18.5 and *micO* RNA are also stimulated, but to a lesser extent (1.2-fold) (Figures 34 and 36). It is therefore concluded that the phosphorylation of RNase III can stimulate RNase III processing activity up to > 11-fold, and that the precise extent of the stimulation is dependent upon substrate sequence.

Does phosphorylation within the dsRBD contribute to the observed stimulation of RNase III processing activity? To test this, a time course study of the RNase III[S195A,S198A] mutant was performed, using R1.1[WC] RNA as substrate. The RNase III samples were pretreated as mentioned above. The RNase III[S195A,S198A] mutant is slightly less reactive, compared to wild-type RNase III (see Table 5, and compare Figure 31, Panel B to Figure 37, Panel A). Thus, the two serines can be changed to alanine without strongly perturbing RNase III function. The activity of the RNase III[S195A,S198A] mutant is stimulated ~2.2-fold upon phosphorylation by T7 PK. We conclude that phosphorylation within the dsRBD as well as the catalytic domain contributes to the overall stimulation of processing activity, and that the majority of the stimulation derives from phosphorylation of the dsRBD.

D. Phosphorylation does not enhance RNase III binding to substrate.

To determine if stimulation of RNase III processing is due to enhanced substrate binding, gel mobility shift assays were performed. In these assays, phosphorylated RNase III[E117K] mutant protein (see **Materials and Methods**) was used, such that substrate binding could be measured in the presence of Mg^{+2} without concomitant cleavage. Increasing amounts of phosphorylated RNase III[E117K], or mock-treated RNase III[E117K], was combined with 5'-^{32}P-labeled R1.1 RNA. The amount of free and bound RNA were measured by radioanalytic imaging unit, and the fraction of RNA bound to RNase III was determined as a function of RNase III concentration. The RNA-protein

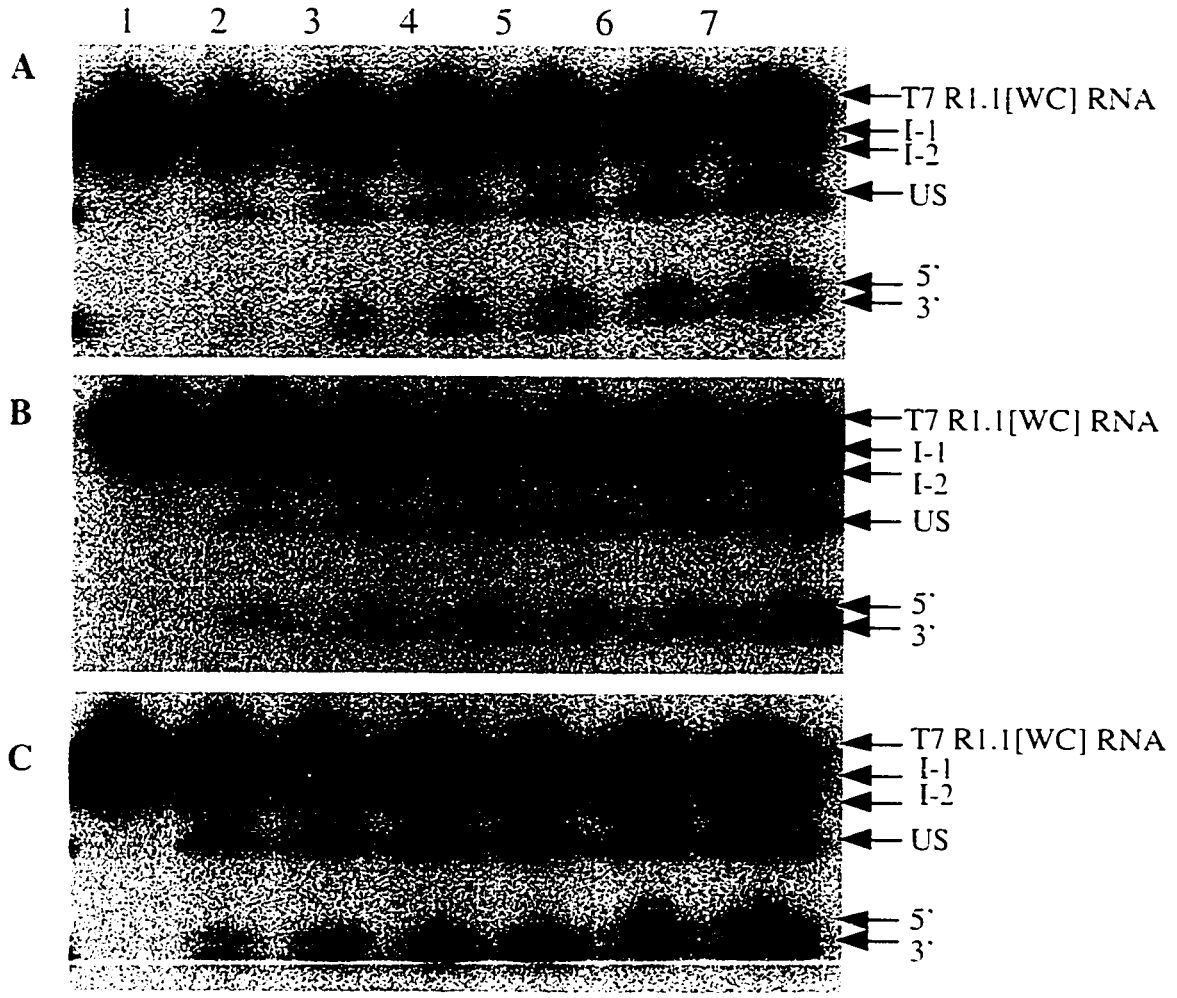


Figure 37. Cleavage of T7 R1.1[WC] RNA by RNase III[S195A, S198A] mutant is enhanced by phosphorylation. Internally ^{32}P-labeled T7 R1.1[WC] RNA (12,000 dpm; 4 pmol) was incubated with RNase III[S195A,S198A] mutant (10nM; 0.2 pmol) at 37^{0}C in cleavage assay buffer for varying times (Lane 1, 0 minute; Lane 2, 1 minute; Lane 3, 2 minutes; Lane 4, 3 minutes; Lane 5, 4 minutes; Lane 6, 5 minutes; and Lane 7, 10 minutes). Reactions were stopped by adding an equal volume of gel electrophoresis sample buffer, then electrophoresed in a 15% polyacrylamide gel (7M urea). Autoradiography was performed using x-ray film and intensifying screens. Positions of uncleaved T7 R1.1[WC] RNA, 5'-end and 3'-end products are indicated with arrows. Also indicated are the products of single-site cleavage (I-1 and I-2). **Panel A:** mock-treated RNase III[S195A,S198A]. **Panel B:** T7 PK[G76F]-treated RNase III[S195A,S198A]. **Panel C:** T7 PK-treated RNase III[S195A,S198A].

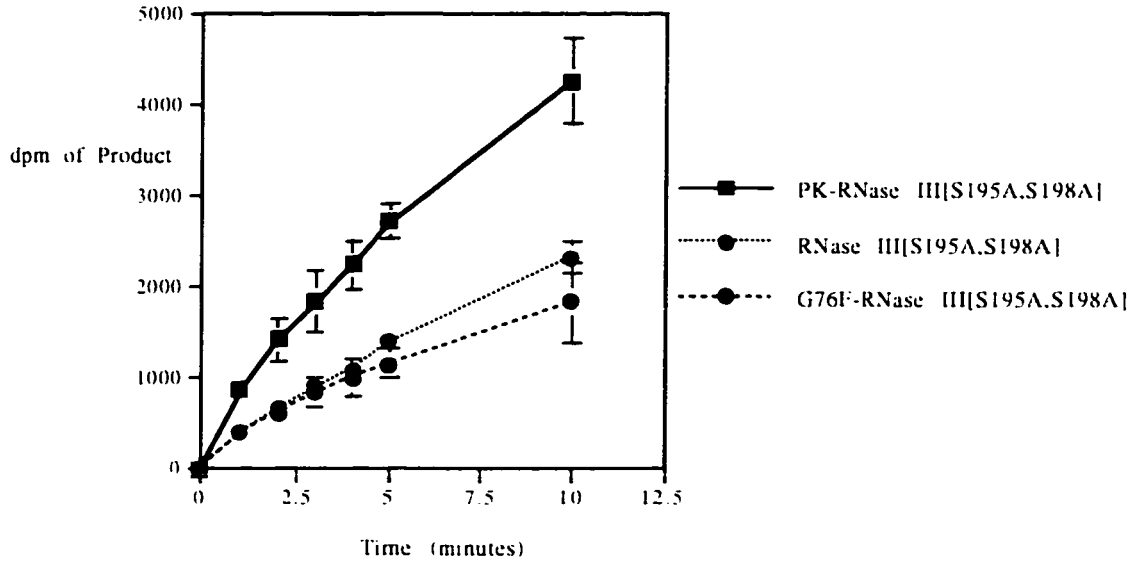


Figure 38. Quantitation of T7 R1.1[WC] RNA cleavage stimulation by phosphorylation of RNase III[S195A,S198A]. Kinetic analysis of T7 R1.1[WC] RNA cleavage. Product amounts were determined by radioanalytic imaging. Black line (PK-RNase III) represents T7 PK-treated RNase III {V_i= 0.29±0.03 pmol min^{-1}}. Red line (RNase III) represents mock-treated RNase III {V_i= 0.13±0.02 pmol min^{-1}}. Green line (G76F-RNase III) represents T7 PK[G76F]-treated RNase III {V_i= 0.13±0.02 pmol min^{-1}}.

Table 5. Initial Velocities (V_i) of Substrate Cleavage by RNase III[a].

	T7 R1.1	T7 R1.1[WC]	T7 R1.1 [WC] [S195A,S198A]	T7 R18.5	*mcO*
PK-RNase III	2.64±0.05 n=8	2.42±0.08 n=8	0.29±0.03 n=4	0.72±0.02 n=4	1.14±0.17 n=4
G76F-RNase III	0.39±0.06 n=8	0.22±0.04 n=8	0.13±0.02 n=4	0.58±0.01 n=4	0.92±0.09 n=4
RNase III	0.41±0.08 n=8	0.22±0.09 n=8	0.13±0.02 n=4	0.59±0.08 n=4	0.91±0.12 n=4
Enhancement[b]	6.8	11	2.2	1.2	1.2

[a]Cleavage rates are reported as pmol min^{-1}

[b]enhancement defined as the V_i (PK-RNase III)/V_i (G76F-RNase III)

complex migrates more slowly than free RNA due to its increased mass. The K'_D (apparent dissociation constant) values were obtained from the slope of a double-reciprocal plot (1/[RNase III(E117K)] vs. 1/fraction of bound RNA) of the data (Figure 39).

The K'_D value for T7 PK-treated RNase III binding to R1.1[WC] RNA is 15.8±0.3 nM. However, there is no increase in the K'_D value, comparing T7 PK-RNase III with G76F-RNase III. Thus phosphorylation does not detectably enhance RNase III binding tosubstrate. There is a ~6-fold increase in T7 PK-RNase III and G76F-RNase III binding to RNA, compared to RNase III. This suggests T7 PK-dependent inhibition of substrate binding by a phosphotransfer-independent mechanism. However, the same relative increase in K'_D value was also observed in mock phosphorylation reactions in which T7 PK is substituted with Bovine serum albumin or T7 RNA polymerase (data not shown). These results indicate that the T7 PK[G76F] stimulation of binding reflects a non-specific effect of protein, and is not specific to T7 PK.

In summary, the results of the gel shift experiments show that there is no enhancement of T7 PK-RNase III binding to substrate, compared to G76F-RNase III binding to substrate. Thus, phosphorylation does not enhance RNase III binding to substrate, and the increased activity of phosphorylated RNase III is due solely to an increase in a step subsequent to binding, which would include the substrate turnover step. Phosphorylation therefore selectively enhances the catalytic rate of RNase III.

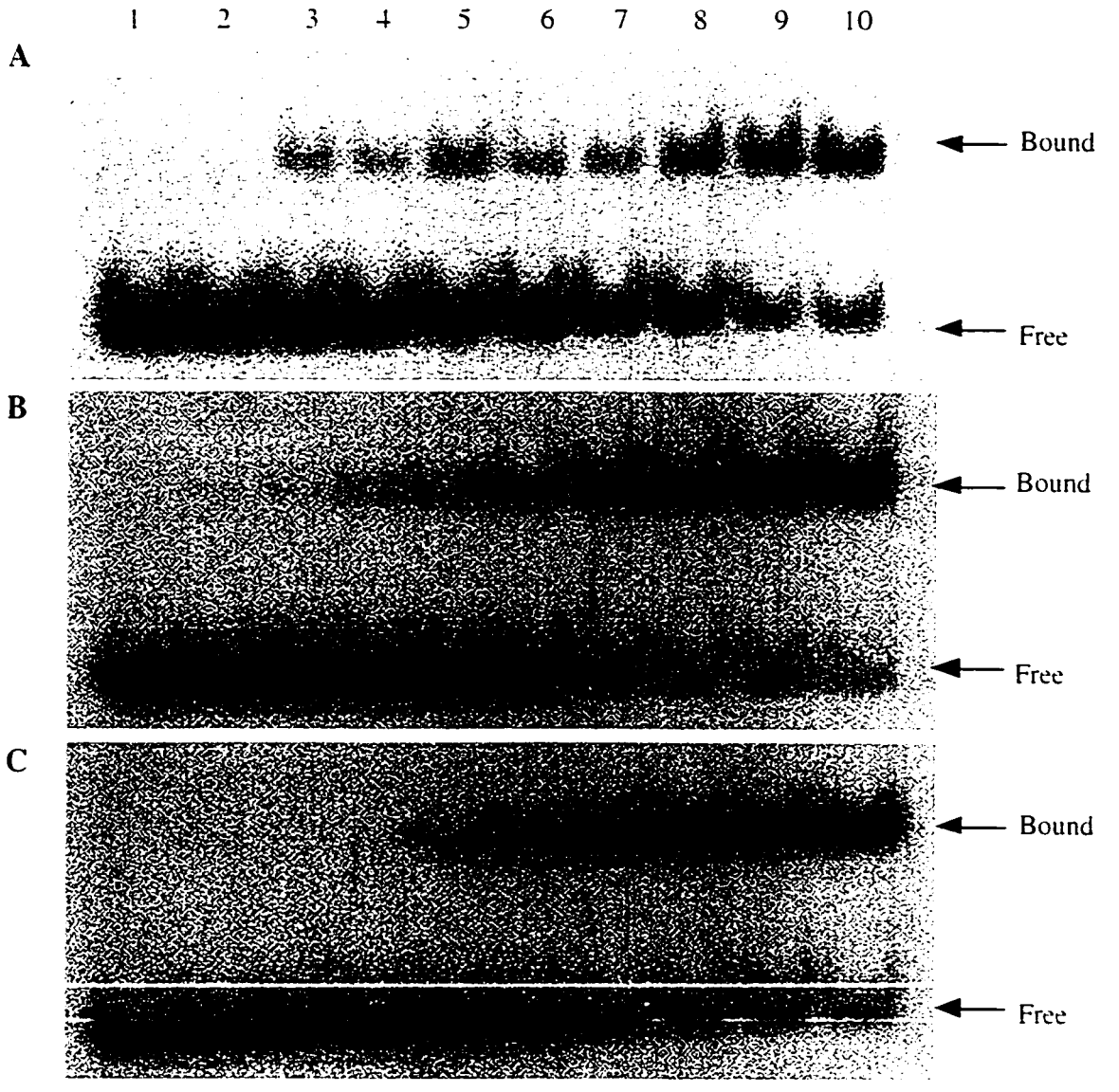


Figure 39. RNase III binding to substrate is not enhanced by phosphorylation. The figure displays an autoradiograms of gel shift assays (non-denaturing 10% polyacrylamide gels). Each lane contains 12,000 dpm of T7 R1.1 RNA (1.8 fmol). Lanes 1,2,3,4,5,6, 7,8,9 and 10 contain 0, 1, 2, 3, 4, 5, 10, 15, 20, and 25nM of RNase III[E117K]. **Panel A:** mock-treated RNase III[E117K]. **Panel B:** T7 PK[G76F] treated-RNase III[E117K]. **Panel C:** T7 PK-treated RNase III[E117K]. Enzyme-RNA complexes are indicated (Bound). Unbound RNA is also indicated (Free).

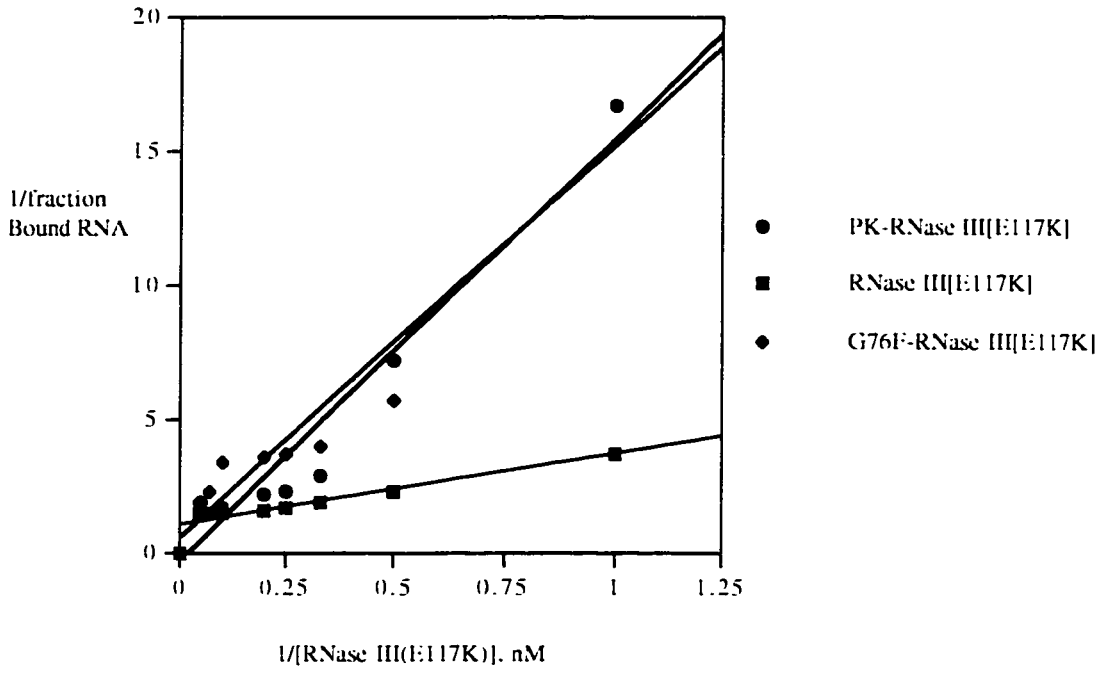


Figure 40. Double reciprocal analysis of T7 R1.1 RNA binding to phosphorylated RNase III. Analysis of results of gel shift assays using T7 R1.1 RNA (see Figure 41). The fraction of RNA bound to RNase III was determined as a function of RNase III[E117K] concentration. K'_D values were determined from the slope. PK-RNase III[E117K]: T7 PK-treated RNase III[E117K] {K'_D=15.8±0.3 nM}. RNase III: mock-treated RNase III[E117K] {K'_D=2.7±1.0 nM}. G76F: T7 PK[G76F]-treated RNase III[E117K] {K'_D=14.7±0.6 nM}.

DISCUSSION

This study has shown that *E. coli* ribonuclease III is phosphorylated by the bacteriophage T7 protein kinase, and that phosphorylation stimulates ribonuclease III activity. Regulation of activity by phosphorylation has heretofore not been demonstrated for any other ribonuclease. Regulation of *E. coli* RNase III activity by phosphorylation provides a paradigm for RNase III homologues, which are ubiquitous among the Bacteria as well as the Eukarya (Gibson and Thompson, 1994). Moreover, regulation of RNase III by phosphorylation may provide a paradigm for understanding the regulation of many other ribonucleases which perform diverse functions, including: angiogenesis, plant antifertility (see below), antitumor, antihelminthic, antiviral, immunosuppressive, neurotoxic, and ribosome-inactivating activities (Sorrentino and Libonati, 1997). There are most likely yet undiscovered functional roles for ribonucleases, and these functions also may be subject to control by covalent modification.

An *in vitro* phosphorylation assay reveals that T7 PK directly phosphorylates RNase III. Thus, the involvement of a secondary protein kinase in the phosphorylation of RNase III *in vivo* can be ruled out. At least two sites within RNase III are phosphorylated. This is evident from the observations that the isolated dsRBD and the catalytic domain are phosphorylated *in vitro*. The phosphorylated residue within the dsRBD is serine 195 and/or serine 198. Within the catalytic domain there are 10 serine residues which are all potential sites of phosphorylation. Serine 46 is located within the highly conserved "signature sequence" of RNase III. To directly determine the phosphorylation sites of RNase III, samples of phosphorylated RNase III have been prepared for analysis by Mass Spectrometry in the laboratory of Douglas Gage (Michigan State University, Department of Biochemistry). Results of these experiments are pending.

Precise determination of the level of phosphorylation of RNase III is complicated by the fact that the number and location of the phosphoserines has not been precisely determined. The level of *in vitro* phosphorylation of RNase III reported in this study is at least 15% (based on pmol phosphate/pmol of RNase III) (see **Results**). The 15% stoichiometry is a minimum estimate, since this number assumes all of the protein added to an SDS-polyacrylamide gel electrophoreses to the same position. In fact there is protein loss, but the precise amount is not known. Indeed, some of the phosphorylated protein is trapped in the wells of the stacking gel (5% polyacrylamide) (data not shown). Whether these proteins are T7 PK, RNase III or both was unable to be determined. Thus, protein aggregation and protein loss suggest a phosphorylation stoichiometry of >20%.

Phosphorylation of RNase III causes a range of stimulation in processing activity, which is substrate-sequence dependent: 11-fold for processing of T7 R1.1[WC] RNA, 6.6-fold for T7 R1.1 RNA, and a slight increase in activity for T7 18.5 RNA and the *rncO* RNA (see **Table 5**). Substrate cleavage and binding assays indicate that phosphorylation by T7 PK directly increases RNase III activity, and there is no other apparent phosphotransfer-independent interactions of RNase III with T7 PK which mediates this stimulation. The level of stimulation in processing activity is apparently dependent upon substrate sequence. The increase in processing activity apparently is accomplished by an enhancement of substrate turnover (k_{cat}), and not from enhancement of substrate binding (as revealed by K'_D values). The increase in RNase III activity is due to enhancement of a step subsequent to binding (probably the turnover step) and not due to enhancement of binding (K_D).

To date, there is only one other report of phosphorylation of a ribonuclease. Kunz and coworkers (Kunz *et al.*, 1996) reported the *in vitro* phosphorylation of three plant self-incompatibility RNases (S-RNases) by the soluble protein kinase Nak-1, which was

partially purified from the pollen tubes of *Nicotiana alata* . S-RNases are located in the extracellular matrix of the plant style and are necessary for gametophytic self-incompatibility (Anderson *et al.*, 1989; McClure *et al.*, 1990). S-RNases are involved in cell-cell recognition in plants, and prevent self-fertilization by entering and degrading mRNA in incompatible pollen tubes. The phosphorylated S-RNases include: S_2-RNase, S_3-RNase, and S_6-RNase. Protein kinase Nak-1 has several features shared by the calcium-dependent class of plant protein kinases (CDPKs). These features include substrate specificity, calcium-dependence, inhibition by calmidazolium, and cross-reaction with monoclonal antibodies raised to soybean CDPK (Roberts and Harmon, 1992). It is not known whether or how phosphorylation alters the activity of the S-RNases.

Phosphorylation has been shown to regulate the activity of RNA-binding proteins. The list of phosphorylated RNA-binding proteins (RNABPs) is more extensive, compared to phosphorylated ribonucleases (Table 6). RNABPs contain one of five major RNA-binding motifs. The major RNA-binding motifs include the RNP domain, the arginine-rich motif (ARM), the RGG box, the KH domain, and the dsRBD (Burd and Dreyfuss, 1994). In some cases phosphorylation occurs within the RNA-binding motif. The covalent modification decreases the affinity of the RNABP for RNA (Fung *et al.*, 1997). In other cases, phosphorylation at one or more sites outside of the RNA-binding motif apparently causes a protein conformational change, which can either increase (e.g., Green *et al.*, 1992) or decrease (e.g., Lisitsky and Schuster, 1995) the affinity of the RNABP for RNA. The chloroplast protein "28RNP" binds to the 3'-ends of chloroplast mRNAs and is involved in mRNA stabilization. Phosphorylation of 28RNP on serine causes a 3-4 fold decrease in affinity for the 3'-end stem-loop structure (Lisitsky and Schuster, 1995). The 28RNP contains three domains, an N-terminal "acidic domain", and two C-terminal "RNA-binding domains". Phosphorylation occurs on a serine residue in the acidic domain and interferes with substrate recognition by the two RNA-binding domains.

Table 6. Phosphorylated RNA-Binding Proteins.

RNABP	Structure	Function	Kinase	Target Amino Acid	Result of Phosphorylation	Reference
28RNP	dsRBD	3'-end processing of chloroplast mRNAs	Casein kinase II	Ser	Affinity for RNA is reduced 3-4 fold	Lisitsky and Schuster (1997)
U4/U6*U5 tri-snRNP	RNP	Nuclear pre mRNA splicing	snRNP kinase	---	---	Fetzer *et al.* (1997)
hnRNP protein A1	RNP	Strand annealing in pre-mRNA splicing	PKA, PKC	Ser	Inhibits Strand annealing activity	Cobianchi *et al.* (1993)
Mei2	RNP	Control of cell cycle	Pat1 kinase	Ser/Thr	Switch from mitotic to meiotic cell cycles	Watanabe *et al.* (1997)
Rex protein	---	Regulates expression of viral structural genes	---	Ser	Increased Binding to viral RNA	Green *et al.* (1992)
AUBF	---	Binds to 3' mRNA UTR	---	---	Increased binding to mRNA 5-7 fold	Malter and Hong (1991)
44kDa and 46kDA RNABP	RGG	rRNA processing and maturation	Casein kinase II	Ser/Tyr	?	Das *et al.* (1996)
hnRNP C1 and C2	---	Splicing of pre-mRNAs	Casein kinase II	Ser/Thr	Inhibits binding of pre-mRNA	Fung *et al.* (1997)
IRP 1 and 2	---	Post-transcriptional regulation of iron uptake	---	---	Increase in to transferrin receptor mRNA 2 fold	Schalinske and Eisenstein (1996)
Rev protein	---	Post-transcriptional regulation of virion gene expression	PKC	Ser	Increase in RNA binding 7-fold	Fouts *et al.* (1997)
Ribosomal protein S6	---	mRNA binding to ribosomes	T7 PK	Ser	---	Robertson *et al.* (1994)
Eukaryotic ribosomal protein S6	---	Control of cell growth and proliferation	Multiple kinases	Ser	Selective translation of mRNAs	Song and Gilbert (1997)
PKR	dsRBD	Anti viral response	PKR	Ser/Thr	Shut off of translation	Wu and Kaufman (1997)
SR proteins	dsRBD	mRNA splicing	SRPK1 and Clk/Sty	Ser	Sequence-specific RNA binding	Tacke *et al.* (1997)

The hnRNP protein A1 is a subunit of the hnRNP complex. Protein A1 binds single-stranded RNA and contains an RNA strand annealing activity. Phosphorylation of protein A1 has no effect on RNA binding affinity, but inactivates strand annealing activity. Protein A1 has two domains, a "single-strand binding domain" at the N-terminus, and a "strand annealing domain" at the C-terminus. Reports indicate that phosphorylation within the C-terminal domain does not alter RNA binding capabilities (Cobianchi *et al.*, 1993). The hnRNP proteins C1 and C2 are also found in hnRNP complexes, and are involved in pre-mRNA splicing. Phosphorylation of these proteins at multiple sites inhibits their binding to pre-mRNAs (Fung *et al.*, 1997).

Among mammalian cells, the double-stranded RNA-dependent protein kinase, PKR (also known as the DAI kinase), is involved in one of the best characterized mechanisms of regulation of protein synthesis. PKR phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF-2α). The subsequent formation of translation initiation complexes is prevented (Dever *et al.*, 1992; Clemens, 1996; Zhu *et al.*, 1997). PKR participates in the antiviral interferon response mechanism (Galabru and Hovanessian, 1985). PKR expression is induced by interferon and its activation is dependent upon binding dsRNA. PKR is a ~69 kDa protein which contains two double-stranded RNA-binding domains at its amino-terminus, and a protein kinase domain at its carboxyl-terminus. The two dsRBDs can bind dsRNA independent of one another; however, both dsRBDs are necessary for maximal binding of dsRNA (Schmedt *et al.*, 1995). Binding of dsRNA by the two dsRBDs activates the kinase activity of PKR. PKR then undergoes autophosphorylation and is then able to phosphorylate its target proteins. PKR occurs in the cytosol as a homodimer, and autophosphorylation can occur in *trans* (Thomis and Samuel, 1993). Phosphorylation of PKR occurs on serine and threonine residues within the region which separates the dsRBDs from the protein kinase domain(Taylor *et al.*, 1996).

Phosphorylation of the *E. coli* RNase III dsRBD at serine 195 and/or serine 198 has no significant effect on the affinity of RNase III for substrate (see **Results**). In contrast, other RNA-binding proteins show a change in RNA-binding properties. Phosphorylation of the adenosine-uridine binding factor (AUBF) (Malter and Hong, 1991), and the iron regulatory proteins 1 and 2 (IRP-1 and -2) (Schalinske and Eisenstein, 1996), cause specific and selective increases in RNA-binding affinities. The RNA-binding affinity of phosphorylated AUBF is selectively increased for the pentameric sequence AUUUA, which is present in multiple copies in the 3'-UTRs of cytokine and lymphokine mRNAs. Binding of phosphorylated AUBF to the 3'-UTRs increases the stability of the mRNA. IRP-1 and IRP-2 are involved in the post-transcriptional regulation of ferritin (an intracellular iron storage protein) and the transferrin receptor (a membrane bound protein which transports iron into the cell). Phosphorylation of IRP-1 and IRP-2 increases affinity for the 3'-end of transferrin receptor mRNA and the 5'-end of ferritin mRNA ~2-3 fold. Binding by IRP-1 and IRP-2 results in an 50% increase in ferritin and transferrin receptor mRNA levels. That the phosphorylation of the RNase III dsRBD might also impart a selective affinity for specific RNAs remains a possibility. Additional substrates would have to be tested in order to assess this.

Phosphorylation of RNase III increases the rate of cleavage of a T7 early processing signal (T7 R1.1 RNA) but does not substantially increase the rate of cleavage of a T7 late processing signal (T7 R18.5 RNA). No increase in cleavage also was observed with a cellular RNase III substrate, *micO* RNA. The cleavage rate enhancement therefore depends in part on specific substrate features. However, the feature(s) which cause the differing enhancement rates is not known.

How might phosphorylation of RNase III be important for the T7 infection strategy? During the early stage of infection, the T7 polycistronic early mRNA precursor is produced

in large amounts. The T7 early proteins are responsible for converting the cytoplasmic environment to one which is optimal for efficient phage reproduction. Since T7 shuts off host gene transcription, and perhaps also host mRNA translation, the levels of RNase III do not increase during T7 infection, and perhaps may even fall. Thus, in order to efficient processing of the polycistronic early mRNA precursor, and the T7 middle and late mRNA precursors, T7 utilizes a protein kinase to directly stimulate the catalytic activity of the limited amount of RNase III. Given the host transcription shutt-off activity, enhanced RNase III processing of cellular RNAs formally is not required for the infection cycle of bacteriophage T7, and the lack of enhanced processing of *micO* RNA is consistent with this proposal. However, additional cellular substrates will need to be tested to see whether the T7 PK stimulation of RNase III is T7 mRNA-specific.

How does phosphorylation of RNase III stimulate catalytic activity without altering substrate binding affinity? Phosphorylation may cause a protein conformational change in or near the RNase III active site. The altered catalytic site may provide enhanced transition state stabilization, thereby lowering the free energy of activation ($\Delta G^{\ddagger}$) without concomitant alteration in substrate binding energy (ΔG_S) (Figure 41). Alternatively, phosphorylation may facilitate product release, if this step is partially rate-limiting (Figure 42). Either model is consistent with an increase in steady-state turnover rate, without an increase in substrate binding affinity. Future enzymological studies should be able to resolve these models.

Future work concerning the regulation of RNase III by the T7 PK should include: a kinetic analysis of RNase III phosphorylated *in vivo*; and the development of an *in vitro* system using the full-length T7 protein kinase (S. Sanders and A.W. Nicholson, in progress). This system would more closely reflect the *in vivo* conditions for RNase III phosphorylation. Mutation of the identified serine target residues to glutamic acid may allow an assessment as to whether the presence of a negative charge at the appropriate sites

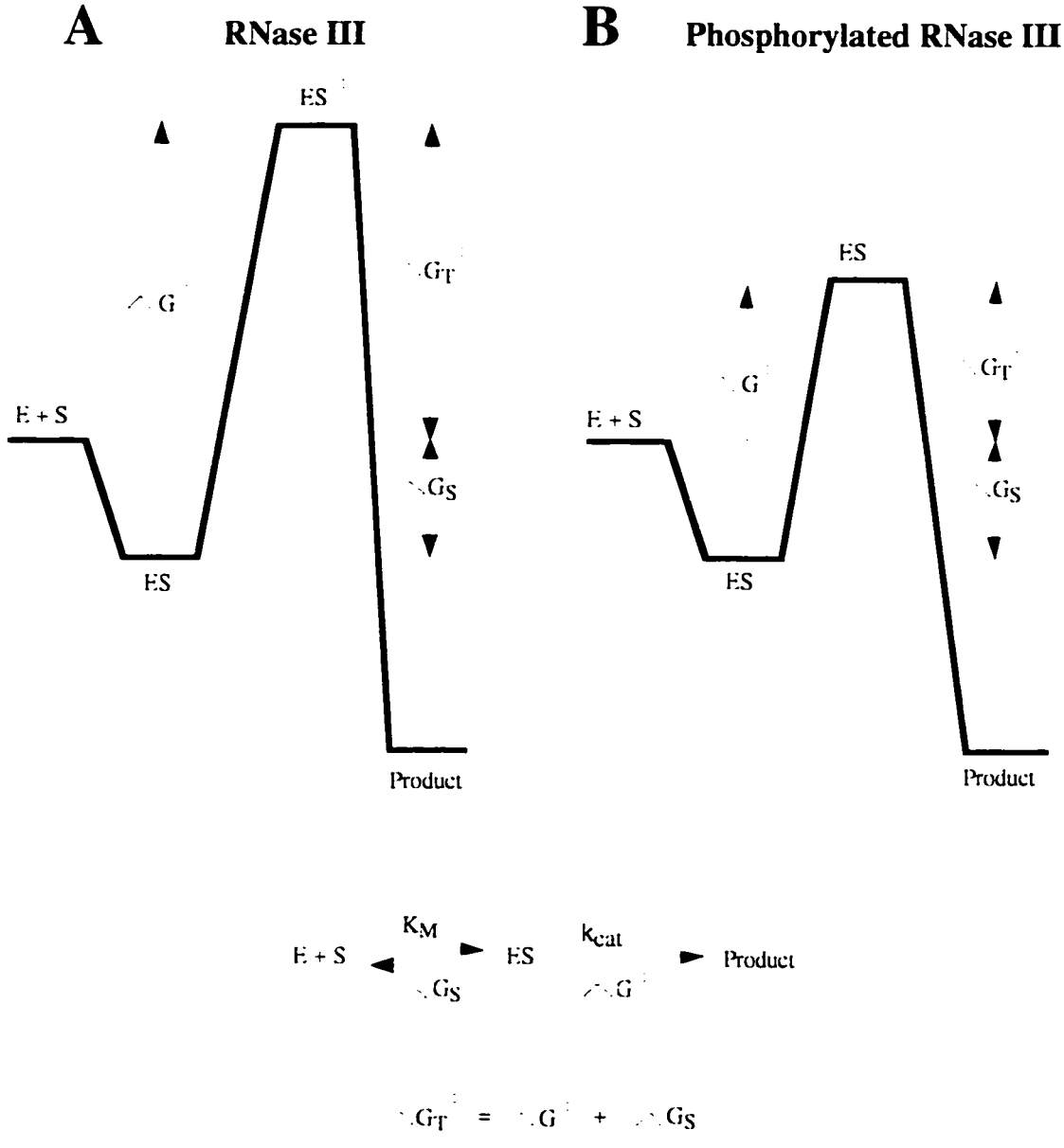


Figure 41. Illustration of Gibbs free energy changes for substrate cleavage by RNase III and phosphorylated RNase III (transition state stabilization). A proposed model of how phosphorylated RNase III is able to increase turnover rate without concomitant increase in binding affinity. **Panel A:** Michaelis-Menten kinetics of RNase III. **Panel B:** Michaelis-Menten kinetics of phosphorylated RNase III. Note that the lowering of the free energy of activation ($\Delta G^{\ddagger}$) in Panel B is accomplished by the lowering of $\Delta G_T^{\ddagger}$ without an alteration in ΔG_S.

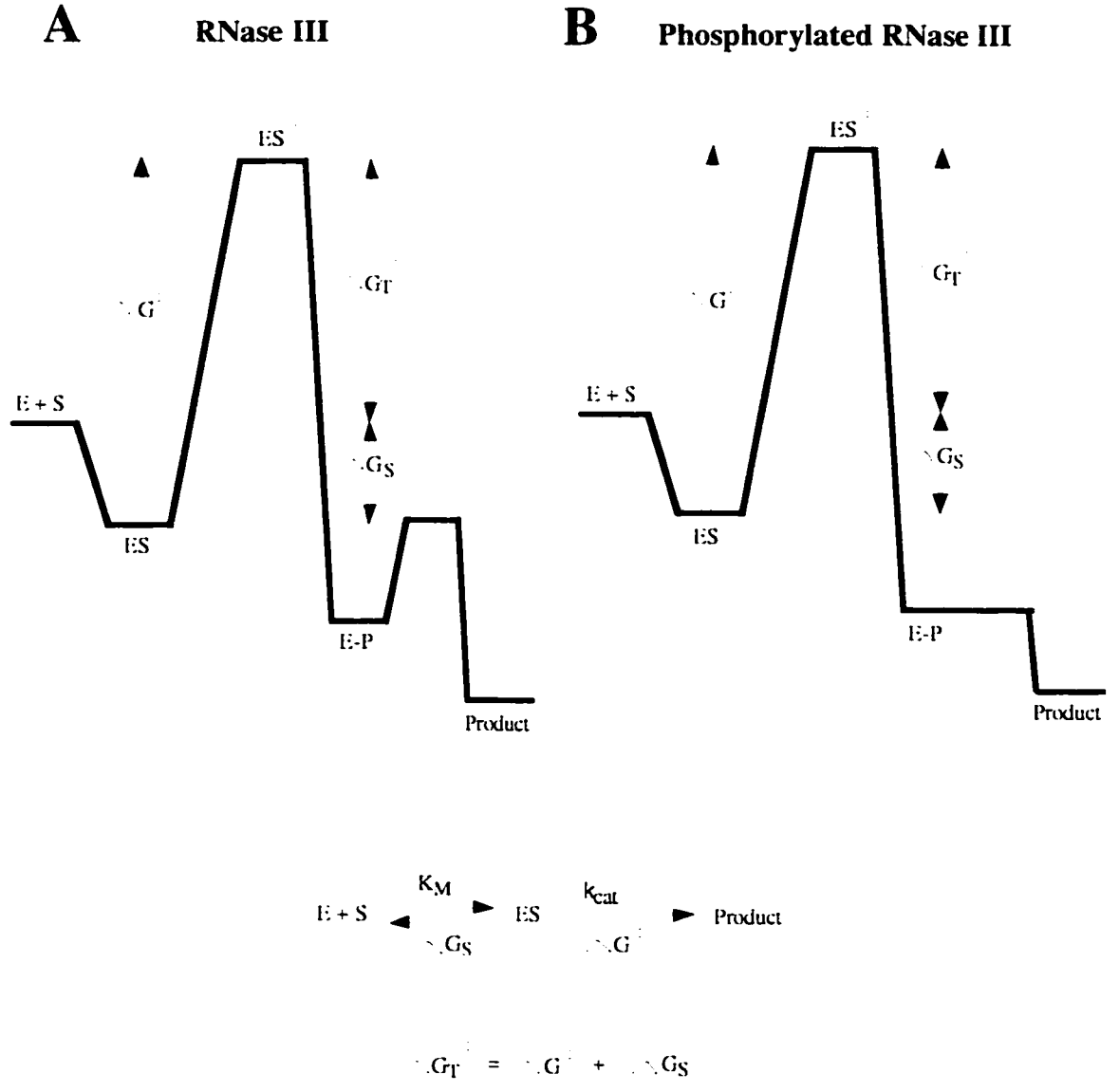


Figure 42. Illustration of Gibbs free energy changes for substrate cleavage by RNase III and phosphorylated RNase III (product release). An alternative model of how phosphorylated RNase III is able to increase turnover rate without concomitant increase in binding affinity. **Panel A:** Michaelis-Menten kinetics of RNase III with an energy barrier to product release. **Panel B:** Michaelis-Menten kinetics of phosphorylated RNase III. If product relase is the rate limiting step, phosphorylated RNase III my adopt a conformation which eliminates this energy barrier.

in the RNase III polypeptide is sufficient to stimulate RNase III activity.

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ABSTRACT

REGULATION OF *ESCHERICHIA COLI* RIBONUCLEASE III BY THE BACTERIOPHAGE T7 PROTEIN KINASE

by

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Ribonuclease III (RNase III) is a double-stranded-RNA-specific nuclease of *Escherichia coli*. RNase III functions as a homodimer. The 25.6 kDa subunit contains two domains, a 148 residue catalytic domain and a 78 residue double-stranded-RNA-binding domain (dsRBD). RNase III is involved in the maturation of the 16S and 23S rRNAs, the degradation of mRNA, and the maturation of T7 phage polycistronic early, middle, and late mRNAs. Bacteriophage T7 expresses a protein kinase (T7 PK), which is cAMP-independent, serine/threonine-specific, and phosphorylates over 90 cellular proteins, including RNase III during infection. RNase III processing activity is stimulated from two to eleven-fold upon phosphorylation by the T7 PK. The increase in processing activity is dependent on substrate sequence, and may be important for the efficient maturation of T7 early mRNAs, which are produced in large amounts and contain several RNase III cleavage sites. The increase in activity reflects an increase in the turnover rate of RNase III. The phosphoamino acid has been determined to be serine. The dsRBD as well as the catalytic domain of RNase III is phosphorylated by the T7 protein kinase *in vitro*.

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ABSTRACTS & PUBLICATIONS

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