

**Cloning, production and site-directed mutagenesis of Bovine Pancreatic  
Ribonuclease A in *Escherichia coli***

A Thesis submitted for the Degree of Doctor of Philosophy of the University of  
London.

by

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**ΕΝ ΟΙΔΑ ΟΤΙ ΟΥΔΕΝ ΟΙΔΑ**

**ΣΩΚΡΑΤΗΣ**

(I only know, I know nothing)

*Socrates*

This Thesis is dedicated to my wife and our children.

## ACKNOWLEDGEMENTS

This thesis is also dedicated to Dr. M.R. Hollaway (deceased), who had the original idea presented in this work. I am also very grateful to Prof. B. R. Rabin who has supervised me through these years with encouragement and wisdom. I thank also Dr. J.M. Ward for useful advice during the first stages of the project. Dr. C. J. Taylorson deserves a special mention for the immense job he has undertaken in the editing of this thesis and also for moral support during the very often, dark times in this work. I would also like to thank Dr. H. J. Eggelte for the synthesis of CpA. I am also grateful to Ms. E. Plummer for the final proof reading. I would like to thank Dr. S. Harbron, Dr. M. Fisher and Mr. D. Marballie for the good and bad times past together. Finally, I would like to mention Ms. E. Sanchez for her help in the beginning of this work, and Ms. A. Theodosiu, which has provided a breathing space for me to be able to finish this thesis.

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## ABSTRACT

The coding sequence for bovine pancreatic ribonuclease A (RNase) precursor has been cloned and produced in *E. coli* using polymerase chain reaction (PCR) techniques.

The use of the coding sequence for RNase precursor to produce mature ribonuclease has proved successful. Pre-ribonuclease is exported to the periplasmic space of *E. coli* and in the process the signal sequence is removed thus producing mature ribonuclease. Formation of the disulfide bridges in ribonuclease is facilitated by the oxidative environment of the periplasm and fully active protein is obtained.

Clones containing DNA coding for mutant enzymes have been obtained using the technique of recombinant circle polymerase chain reaction (RCPCR). This work was carried out to test the applicability of RCPCR for the production of mutant ribonucleases, and the RCPCR technology is now validated for this purpose.

The kinetic constants of these mutants have been investigated using dinucleotide substrates. The findings are discussed in terms of the interaction of active site residues with dinucleotide substrates.

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## ABBREVIATIONS

- $\epsilon$ : extinction coefficient
- $\mu\text{l}$ : microliter ( $10^{-6}$ )
- $\mu\text{m}$ : micrometer ( $10^{-6}$ )
- $\Delta\text{OD}_{x \text{ nm}}$ : Change in optical density at x nanometres.
- A: Alanine
- Å: Amstrong
- Annealing buffer (10x): 100 mM Tris/HCl pH 8.8, 1 M NaCl, 10 mM  $\text{Na}_2\text{EDTA}$
- bp: base pair
- C > p: Cytidine-2':3'-Cyclic phosphate
- cDNA: complementary DNA produced by reverse transcribing mRNA
- CIP: Calf intestine alkaline phosphatase
- CpA: Cytidylyl (3'→5')-Adenosine
- CpC: Cytidylyl (3'→5')-Cytidine
- CpG: Cytidylyl (3'→5')-Guanosine
- CpU: Cytidylyl (3'→5')-Uridine
- E: Glutamic acid
- *E*: optical density
- FPLC: Fast Protein Liquid Chromatography
- g (ave): average centrifugal force
- IPTG: Isopropyl- $\beta$ -D-Thiogalactopyranoside
- K: Lysine
- Kb: kilobase or 1000 bases
- *lac* promoter: Forms part of the lactose operon
- Lysis buffer: 0.9% (w/v) glucose, 50 mM Tris/HCl pH 7.5, 1 mM  $\text{Na}_2\text{EDTA}$
- MES: 2-[N-Morpholino] ethane sulphonic acid
- Met-bGH: Methionyl bovine growth hormone
- mRNA: messenger RNA
- N: Asparagine
- nm: nanometer ( $10^{-9}$ )



- OD: optical density
- PCR: Polymerase Chain Reaction
- PEG 6000: Polyethylene glycol 6000
- **pfu**: A thermostable DNA polymerase from *Pyrococcus furiosus*
- pmols: picomols ( $10^{-12}$ )
- PolIk: DNA polymerase I (Klenow fragment)
- pre-RNase: The precursor form of ribonuclease
- Q: Glutamine
- R: Arginine
- RBS: Ribosome binding site
- RCPCR: Recombinant Circle Polymerase Chain Reaction
- RNase: Bovine pancreatic ribonuclease A
- S-Sepharose FF: Sulphonic acid-Sepharose Fast Flow
- SDM: Site-directed mutagenesis
- SDS-PAGE: SDS- Polyacrylamide gel electrophoresis
- SDS: Sodium dodecyl sulphate
- Stop mix (5x): 0.1 M Na<sub>2</sub>EDTA, 40% (w/v) sucrose, 0.15 mg/ml Bromophenol blue
- *tac*promoter: A hybrid of the *trp* and *lac* promoters
- TE buffer: 10 mM Tris/HCl pH 7.5, 1 mM Na<sub>2</sub>EDTA, 20 µg/ml RNase (optional)
- TEAA: Triethylamine acetic acid
- TEMED: N,N,N',N'-Tetramethylethylenediamine
- Tet/Z: A thermostable DNA polymerase from *Thermus thermophilus*
- Tris base: Tris (hydroxymethyl) aminomethane
- Tris/HCl: Tris (hydroxymethyl) aminomethane hydrochloride
- *trp* promoter: Forms part of the triptophan operon
- UV: Ultraviolet
- X-Gal: 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
- YM-10: Molecular weight cut off > 10000.

## CHAPTER 1: INTRODUCTION

"Pancreatic ribonuclease A: the most studied endoribonuclease". This is the title of a review on ribonuclease by Eftink and Biltonen (1987). Since it was first discovered in 1920 (Jones, 1920), an enormous amount of information has been available in the literature pertaining to the enzyme. In this chapter, emphasis has been put on the mode of action of bovine pancreatic ribonuclease A (RNase), since the work described in the following chapters is directed to its understanding. The materials and methods used to accomplish this work are described in chapter 2. The design of an expression system in *Escherichia coli* to produce recombinant RNase is explained in chapter 3. An account of the production of mutants of ribonuclease using a technique which involves the polymerase chain reaction is given in chapter 4 and the kinetic characteristics of mutants so produced is presented in chapter 5.

An overview of the literature pertaining to RNase, with a marked emphasis on the mechanism of action is presented in this chapter followed by an account of current strategies for the expression of exogenous proteins in prokaryotes and techniques for altering residues of these proteins by site-directed mutagenesis.

### 1.1 Biological role of RNase

Ribonucleases are a class of enzymes which are found in most species (Beintema, 1990).<sup>(see Appendix II)</sup> One of the best studied ribonucleases is the one pertaining to the cow. Bovine pancreatic ribonuclease A (RNase, EC 3.1.27.5) contains 124 amino acids and is an endoribonuclease which has been characterized extensively. It is produced in the pancreas as a pre-enzyme (150 amino acids), requiring cleavage of the leader sequence prior to correct folding and export to the rumen where the enzyme is active (Barnard, 1969a). Its function is the degradation of microbial ribonucleic acid (Barnard, 1969b). In other vertebrates, including man, RNase is found at very low levels and its function is unclear but could involve the breakdown of dietary RNA

(Barnard, 1969b). Enzymes with ribonucleolytic activity are also found in prokaryotes (Sevcik *et al.*, 1990).

## 1.2 Structure of RNase

The structure of RNase has been elucidated using X-ray crystallography at a resolution of 1.45 Å (Borkakoty *et al.*, 1982). The data obtained made it possible to propose the following structural motifs: a) the protein is U-shaped with approximate dimensions of 35 x 45 x 31 Å, 2) the active site is located in a pronounced cleft and 3) there are three segments of helix; residues 3 to 13, residues 24 to 33 and residues 50 to 60, the latter two segments contain a rare  $3_{10}$  helix as well as an  $\alpha$  helix, and 3 antiparallel  $\beta$ -structures between residues 42-48, residues 71-92 and residues 94 to 110.

## 1.3 Structure and function of RNase A binding subsites

RNase mainly hydrolyzes single stranded RNA. Extensive double stranded structure and tertiary structure in tRNA may render many regions initially insusceptible to RNase (Billeter *et al.*, 1966). However, short helical domains in RNA (<15bp) are apparently insufficient in themselves to confer high resistance (Gould *et al.*, 1966), presumably because the ends readily open up. The presence of subsites in RNase for the binding of RNA, may confer on the enzyme the ability to attach itself to one strand of a double helix, leaving the other susceptible to degradation by another molecule of RNase (B. R. Rabin, personal communication).

Wellner and colleagues (1963) have demonstrated that by attaching polypeptides of alanine to the free amino groups of RNase, the enzymatic activity of the modified enzyme towards RNA is considerably decreased but remains equal to that of the unmodified enzyme when using small molecular weight substrates (i.e. cytidine 2':3' cyclic phosphate) (see Fig. 1.2). This strongly suggests that the amino groups in RNase affect RNA binding, but not hydrolysis of small substrates.

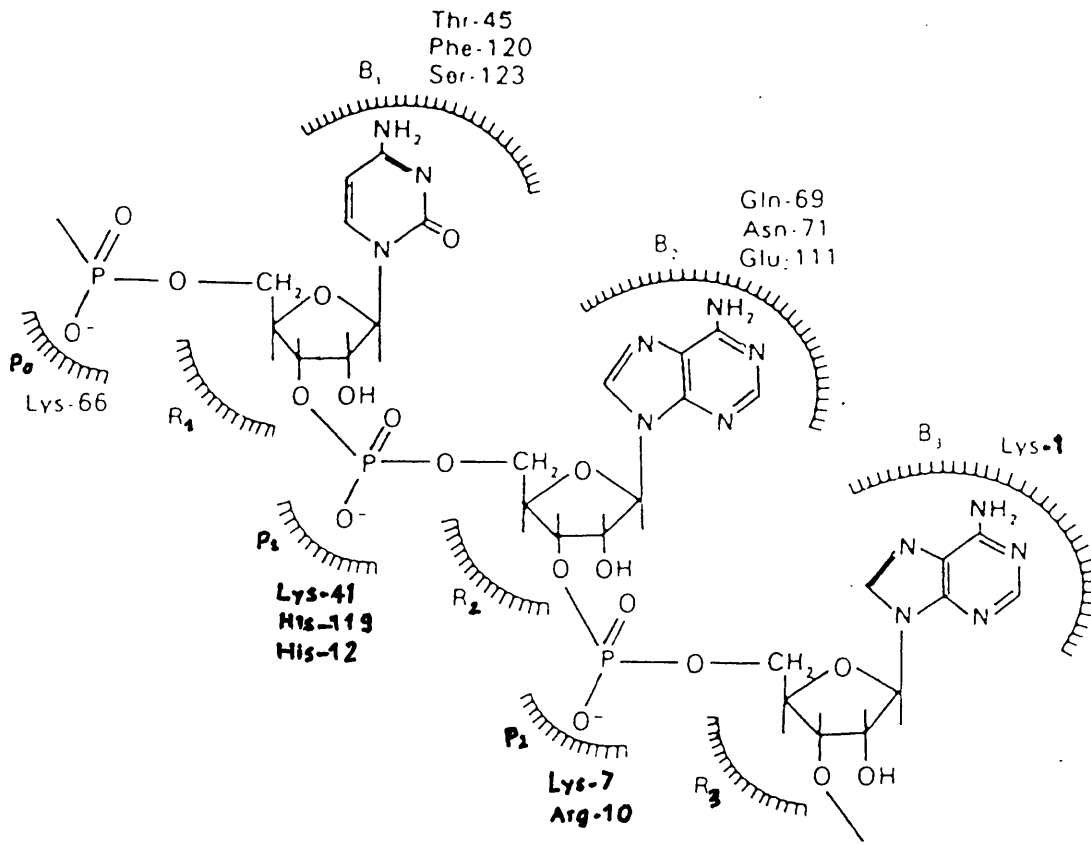
Several subsites have indeed been found in RNase whose function is the anchoring of RNA so that the effective substrate concentration increases in the active site. The nomenclature of these sites is described in Fig. 1.1 (Pares *et al.*, 1991).

The B<sub>1</sub> subsite is always occupied by a pyrimidine and the B<sub>2</sub> subsite can be filled by either a purine or a pyrimidine, however the nature of the base in the B<sub>2</sub> subsite affects the catalytic rate with which the enzyme turns over the substrate. The kinetic parameters for a series of (3'-5') dinucleotides substrates of the form CpN (where N can contain any one of the four bases) have been determined by Witzel *et al* (1962) (see table 1.1). Depending on the nature of the base N, the  $k_{cat}$  varies a 100-fold while the  $K_m$  is hardly affected. This phenomenon is not yet understood but suggests that specific interactions between the enzyme and the base associated with N are formed when the preferred bases are occupying the B<sub>2</sub> subsite.

The amino acids that form the B<sub>1</sub> subsite are Thr-45, Ser-123 and Phe-120 (Llorens *et al.*, 1989). Borkakoti (1983) crystallized enzyme-inhibitor complexes and postulated that the pyrimidine nucleotides bind at the active site (via atoms O2 and N3) at only two locations on the protein involving the residue Thr-45. Purine nucleotides cannot bind in the same way as pyrimidines due to steric hindrance and charge incompatibility and interact with the B2 subsite of the enzyme.

The amino acid Ser-123 was predicted to be near the active centre by Richards and Wyckoff (1971), however the replacement of this amino acid by alanine does not substantially affect the substrate binding properties of the Ala derivative (Hodges and Merrifield, 1974). In contrast to Thr-45, Ser-123 is not conserved in RNases from other mammalian species. Therefore, the nature of the amino acid at position 123 is not crucial for base recognition.

Lin *et al.*, (1972) have carried out extensive work to elucidate the role of Phe-120. The main role of this amino acid is to help in the maintenance of RNase in a compact



**Figure 1.1:** Schematic diagram of the subsites in RNase.

B, R and p indicate binding subsites for base, ribose and phosphate, respectively. B<sub>1</sub> is specific for pyrimidines and B<sub>2</sub> "prefers" purines. The phosphate group of the phosphodiester bond hydrolysed by the enzyme binds to p<sub>1</sub>. The residues known to be involved in each site are as indicated.

**Table 1.1:** Kinetic constants obtained for RNase at pH 7.0 (imidazole buffer), for the substrates cytidylyl- (3'-5')-adenosine (CpA), cytidylyl-(3'-5')-guanosine (CpG), cytidylyl-(3'-5')-cytidine (CpC), cytidylyl-(3'-5')-uridine (CpU) and cytidine-2':3'-cyclic phosphate (C>p) (Witzel *et al.*, 1962).

	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ (mM)	$k_{cat}/K_m$ (mM <sup>-1</sup> s <sup>-1</sup> )
CpA	3000	1.0	3000
CpG	500	3.0	165
CpC	240	4.0	60
CpU	27	3.7	7
C>p	5.5	3.3	1.5

structure as evidenced by the fact that native RNase is very resistant to trypsin digestion. If phenylalanine 120 is removed, the mutant enzyme becomes highly susceptible to attack by trypsin. The amino acid Phe-120 also helps in the positioning of His-119, an important amino acid for catalysis, (see 1.4, Fig. 1.2) in the most favourable position for interaction with the substrate. Removal of the last 4 amino acids of RNase (-Asp-Ala-Ser-Val-OH) yields an enzyme with low but definite activity, while the further loss of Phe-120 gives an enzyme with no activity at all. However, Phe-120 does not seem to be involved in substrate binding, since its replacement by leucine or isoleucine yields derivatives that have similar  $K_m$  for cytidine 2':3'-cyclic phosphate (see Fig. 1.2). These studies strongly implicate Thr-45 as the amino acid responsible for the specificity for pyrimidines in the  $B_1$  subsite.

The  $p_o$  subsite is composed mainly of Lys-66. Upon binding of inhibitors by the enzyme, the flexibility of the side chain of Lys-66 is greatly reduced and an approach of Lys-66 towards the C5'-OH group of the sugar (in the  $R_1$  position) of the bound nucleotide is observed (Borkakoti, 1983). This supports the concept that the side chain of Lys-66 is involved in the binding of 5'- phosphates of inhibitors bound at the  $B_1$  subsite (Mitsui *et al.*, 1978).

The  $R_1$  subsite is near His-12, Lys-41, Val-43, Asn-44 and the peptide bond between amino acids 120 and 121 (Llorens *et al.*, 1989). Lys-66 interacts with the ribose in the  $R_1$  subsite as described above.

The phosphate linking the ribose at the  $R_1$  subsite and the ribose at the  $R_2$  subsite (3'-5' direction) forms part of a well characterized site named the  $p_1$  subsite. The amino acids His-12 and His 119 and the peptide bond between the latter and Phe-120 form part of this subsite and Gln-11 and Lys-41 are also nearby (Llorens *et al.*, 1989). However, the work of Borkakoti *et al.*, (1983) has indicated that Lys-41 is not involved directly in the primary recognition of the phosphate but in the stabilization of the pentacoordinated cyclic phosphate intermediate (see 1.4). This is corroborated by

the data presented in chapter 5 where the  $K_m$  of a mutant RNase, where Lys-41 has been replaced by an Arg, for the dinucleotide substrate CpA is similar to that of the native enzyme. However, this mutant (K41R) has a very low  $k_{cat}$  when compared to native RNase for the same substrate (see 5.3).

The ribose 5' to the phosphate is located in the  $R_2$  subsite and the aminoacids comprising it are; Ala-4, Phe-8, Val-118 and the peptide bond between Val-118 and His-119 (Llorens *et al.*, 1989).

The leaving group (i.e a purine or a pyrimidine) is located in the  $B_2$  subsite. An extensive description of this subsite is presented in chapter 5. The available literature suggests that this subsite is formed by the amino acids Gln-69, Asn-71 and Glu-111 which directly interact with the base.

The  $p_2$  subsite is located close to Lys-7 and Arg-10 (Richardson *et al.*, 1990). Other residues near this subsite are; Glu-2, Thr3 and Ala-4 (Llorens *et al.*, 1989). The ribose occupying the  $R_3$  subsite is near Lys-7, Arg-10, Gln-11 and Leu-35 (Llorens *et al.*, 1989). The  $NH_2$  of the third base in the  $B_3$  subsite lies close to  $-NH_2$  of Lys-1. Amino acids Glu-2, Thr-3, Arg-10, Asn-34, Asp-38 and Arg-39 also form part of the  $B_3$  subsite (Llorens *et al.*, 1989). The residue Lys-1 has been implicated in binding the substrate  $ApA > p$ , which interacts in  $B_2$ ,  $p_2$ ,  $B_3$ ,  $p_3$  (Irie *et al.*, 1986). However, McPherson *et al.*, (1986) using the substrate  $d(pA)_4$  did not find a direct involvement of Lys-1 with the phosphate of this substrate. Therefore, the role of Lys-1 remains somewhat unclear.

### 1.3.1 Role of lysines or positive areas of the protein

Llorens *et al.*, (1989) have indicated that the enzyme consists of at least five positive regions for the binding of RNA. The primary recognition event would be a non-specific electrostatic interaction between five phosphate groups of the RNA and five positive regions in the enzyme. This would direct the correct alignment of the



substrate molecule in the active site allowing the establishment of specific interactions that would lead eventually to the hydrolysis of RNA.

These positive areas of the enzyme which are responsible for RNA binding are in accordance with the experiments reported by Sela (Wellner *et al.*, 1963, Frensdorff *et al.*, 1967, and Frensdorff and Sela, 1967). In these studies polypeptide chains were attached to the free amino groups of the enzyme thus producing enzyme derivatives with impaired RNA hydrolysis. However, the hydrolysis of small substrates like cytidine 2':3'-cyclic phosphate (see 1.4, Fig. 1.2) was maintained and even in some cases the activity of the enzyme towards this substrate was increased when compared to the activity shown by the native enzyme.

All these experiments were carried out in phosphate buffer (Frensdorff *et al.*, 1967), thus the essential residue Lys-41 was protected from reacting with the different polypeptide chains used.

#### 1.4 The active site and mechanism of action of RNase

It is clear that the complexes formed between RNA and RNase involve several nucleotide units interacting at appropriate subsites on the enzyme. Although all of these may have a role in the proper orientation of the substrate with respect to the catalytic entities, the subsites that interact with the bases associated with nucleoside residues directly bonded to the phosphorous atom in the chemical process (subsites B<sub>1</sub> and B<sub>2</sub>) have a crucial dominating effect on the catalytic process. This must be true because values of  $k_{cat}/K_m$  of 2700 mM<sup>-1</sup> s<sup>-1</sup> are observed for the best dinucleotide substrate (CpA) (see chapter 5). The use of dinucleotide substrates as a model for studying the mechanism of action of RNase is then validated.

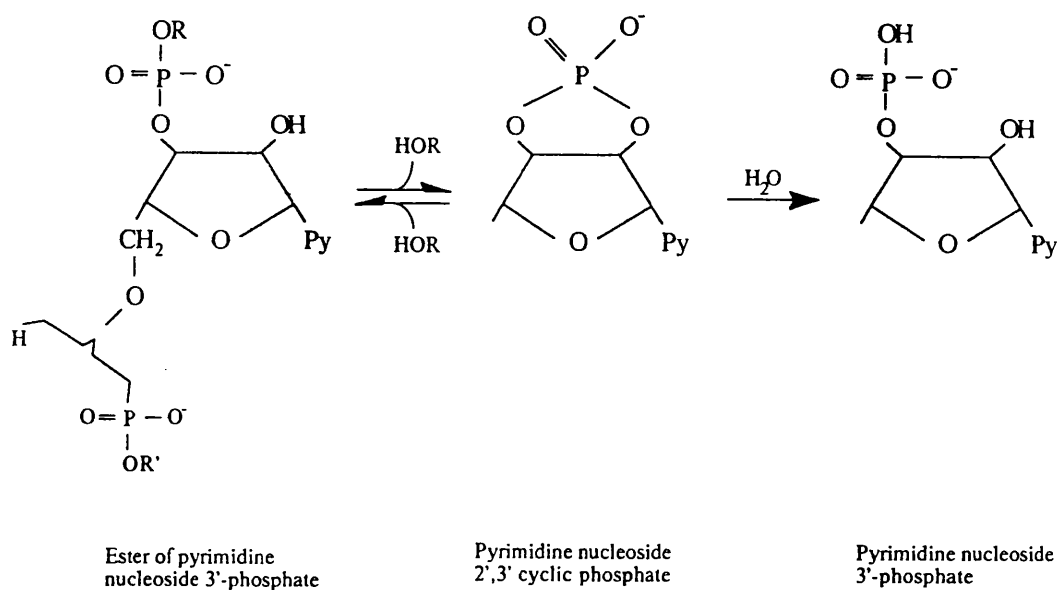
The active site of the enzyme is composed of the subsites B<sub>1</sub>, R<sub>1</sub>, p<sub>1</sub>, R<sub>2</sub> and B<sub>2</sub> (see Fig. 1.1). The amino acids which form part of these subsites have already been described in section 1.3.

Bovine pancreatic ribonuclease A (RNase) catalyzes the hydrolysis of 3'-5'-phosphodiester linkages of single stranded RNA at the 5' ester bond in a two step reaction (see Fig.1.2). The first step is a transphosphorylation or cyclization reaction which yields a 2':3'-cyclic phosphate terminus and a free 5'-OH group on the other side of the bond cleaved. The second step is the hydrolysis of the cyclic phosphodiester intermediate to give a 3'-phosphate monoester. The base at the 3' side must be a pyrimidine (cytosine or uracil) and occupies the B<sub>1</sub>, R<sub>1</sub> and p<sub>1</sub> subsites. The base at the 5' side can be either a pyrimidine or a purine and occupies the R<sub>2</sub> and B<sub>2</sub> subsites.

The original mechanism (Findlay *et al.*, 1961) suggested that catalysis involved a pair of histidine residues. These two histidine residues are His-12 and His-119; in the cyclization reaction, one of them acts as a base (His-12) and the other as an acid (His-119) in what is an acid-base driven mechanism for the hydrolysis of RNA or dinucleotide (3'-5') substrates. During hydrolysis of the cyclic intermediate, the roles of the histidines are reversed.

The amino acid Lys-41 has been found to be implicated in the reaction pathway (see chapters 4 and 5 and references therein), its replacement by arginine using site-directed mutagenesis yields a mutant enzyme that has a reduced activity for RNA hydrolysis (chapter 4) and a large reduction in  $k_{cat}$  when CpA is used as substrate when compared to the respective activities of native and recombinant RNase (see Chapter 5) . This indicates that its possible role rather than binding of the substrate is that of favouring the catalytic mechanism by interacting with the phosphate moiety during the reaction process. Rabin (1990) has indicated that Lys-41 has a flexible interaction with one negatively charged oxygen atom attached to the phosphorous group of the substrate (see Fig. 1.5).

Another amino acid thought to be implicated in the mechanism is Asp-121. The role of Asp-121 is not clear. Raines, (1990) replaced this amino acid by Ala using site-



**Figure 1.2:** The two step hydrolysis of RNA by RNase.

In the transphosphorylation step a cyclic phosphate intermediate is formed which is hydrolyzed in the subsequent step.

directed mutagenesis causing a 10-fold reduction in enzymatic activity. Stern and Doscher, (1984) produced a semisynthetic enzyme formed by the non-covalent interactions of residues 1-118 of RNase and a synthetic tetradecapeptide containing residues 111-124. The replacement of Asp-121 by Asn produced a derivative that is 4.5% active against cytidine 2':3'-cyclic phosphate. This reduced activity results entirely from a diminished catalytic efficiency and not from a decreased affinity for the substrate.

#### **1.4.1 Chemical mechanism for the RNase catalytic reaction**

During the transphosphorylation or cyclization step, His-12 acting as a general base takes a proton from the 2'-OH on the ribose. Then, a nucleophilic attack takes place on the phosphate while His-119 acting as a general acid donates a proton to the 5' oxygen of the leaving group, thus releasing it (see Fig. 1.3). In the hydrolysis reaction, the roles of the histidines are reversed. His-119 (acting base) takes a proton from a water molecule, the free hydroxyl attacks the phosphate as His-12 (acting acid) protonates the 2' position on the ribose (see Fig. 1.3). If a nucleoside with a free 5' hydroxyl replaces water, the reversal of the first reaction occurs.

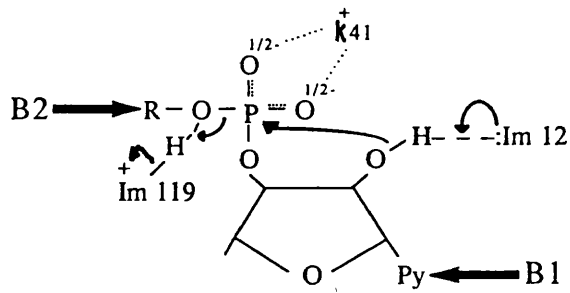
It has been postulated that the reaction goes through a pentacovalent state. In the currently accepted mechanism, this is taken as a transition state. In the mechanism of cyclization shown in Fig. 1.3, the bond between the attacking 2' oxygen and the phosphorous atom forms as the bond between the leaving 5' oxygen and the phosphorous breaks. The converse happens in the hydrolysis of the cyclic. For both of the reactions catalyzed, the attacking and departing oxygens would be in the apical position of a notional trigonal bipyramid as shown in Fig. 1.4. The other two oxygens are stabilized by Lys-41.

However, there are authors that do not believe in the stabilization of transition states because of the intrinsic nature of it, therefore a new mechanism has been proposed by Rabin (1990) (see Figs. 1.5a and 1.5b). The mechanism proposed involves the

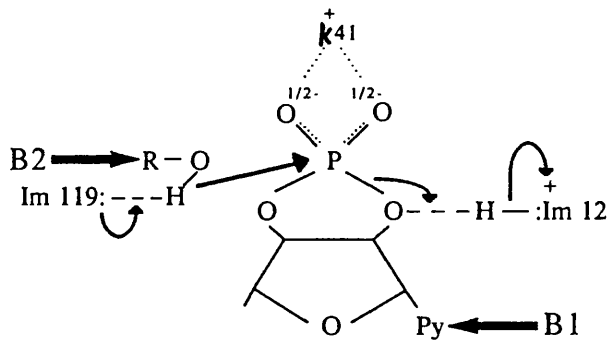
formation of pentacovalent molecules as true intermediates, neither of these being a transition state as shown in Fig. 1.4.

Whether the hydrolysis of dinucleotides proceeds through a "transition state" in the form of a notional trigonal bipyramid or this same form is treated as a true intermediate (with a transition state possibly necessary to form it) is something that at the moment can not be elucidated.

(a) Cyclisation reaction

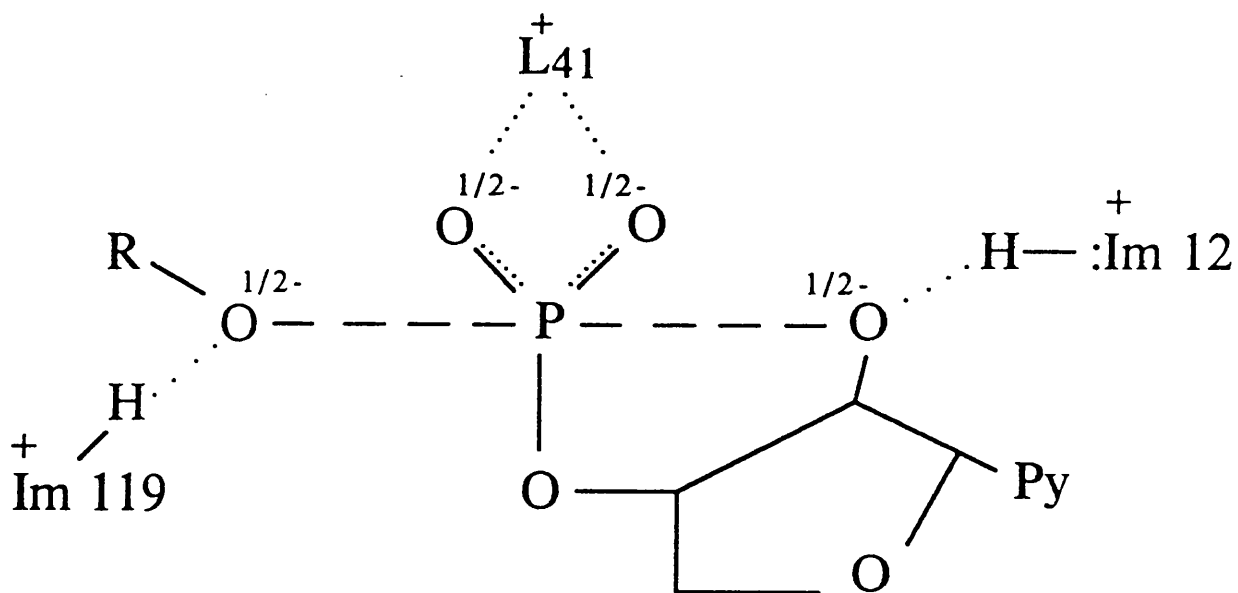


(b) Decyclization reaction



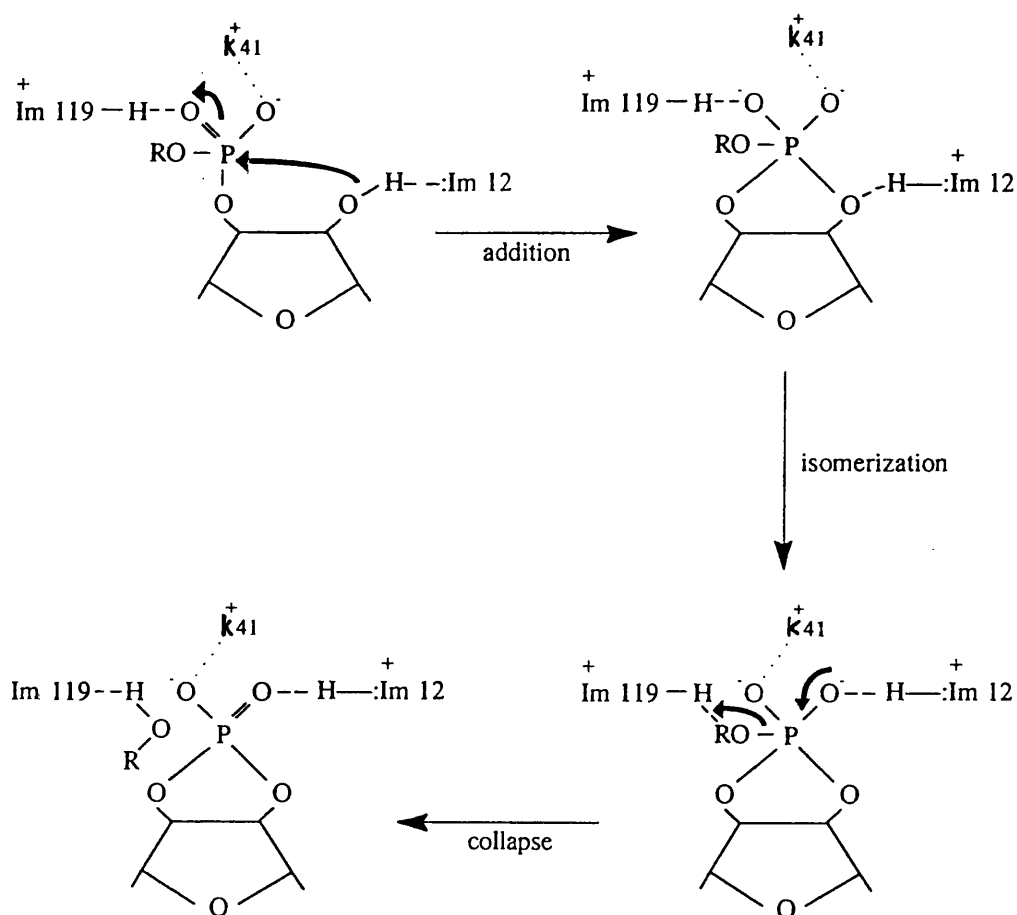
**Figure 1.3:** Mechanism of RNase for the <sup>transesterification</sup> of RNA.

The original mechanism proposed by Findlay and collaborators (Findlay *et al.*, 1961) suggested that catalysis involved a pair of histidines (His-12 and His-119). In the cyclisation reaction (a), one of them functions as a base (His-12) and the other as an acid (His-119) in an in-line nucleophilic displacement process. During hydrolysis or decyclisation of the cyclic phosphate intermediate, the roles of the respective histidines are reversed.



**Figure 1.4:** Proposed "transition state" for the mechanism of hydrolysis of RNA shown in Fig. 1.3.

(a) Cyclization reaction



**Figure 1.5a:** New mechanism for the <sup>transesterification</sup> of RNA by RNase proposed by Rabin (1990).

In the cyclisation reaction, His-119 is positioned so as to act as an effective acid catalyst for an initial nucleophilic addition of the 2'- oxygen to the phosphorous catalysed by His-12 acting as a base. The addition process produces the first pentacovalent intermediate, which undergoes an isomerisation reaction. Following isomerisation, acid catalysis by His-119 causes the second pentacovalent intermediate to collapse, releasing the leaving group HOR. The decyclisation or hydrolysis of the cyclic phosphate intermediate is shown in Fig. 5b.



(b) Decyclization reaction

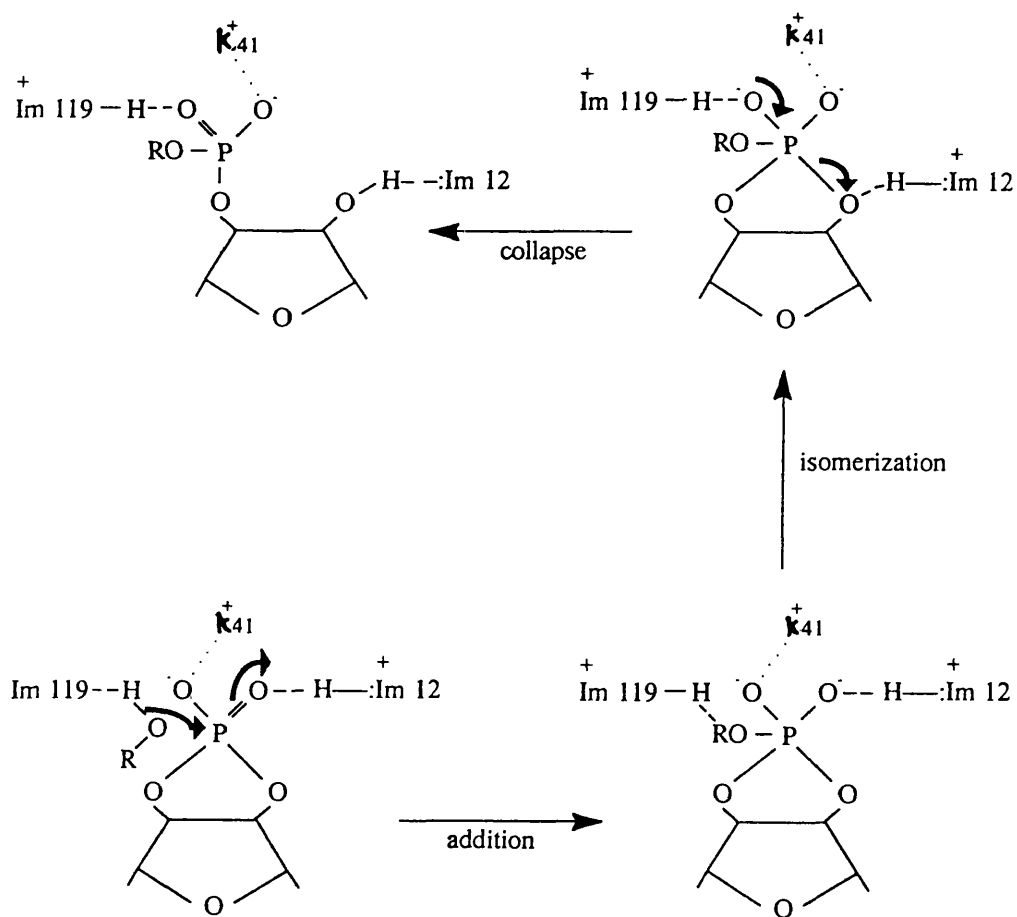


Figure 1.5b: Proposed mechanism for the decyclisation reaction.

## 1.5 Ribonuclease related proteins

### 1.5.1 Angiogenin

Human angiogenin is a blood-vessel inducing protein whose primary structure displays a 35% identity to that of RNase. Angiogenin also catalyzes limited cleavage of 18-s and 28-s ribosomal RNA and is several orders of magnitude less potent than RNase toward conventional substrates (i.e. polycytidylyl or dinucleotide substrates) (Harper and Vallee, 1989).

Most of the aminoacids pertaining to the active site of RNase are conserved in angiogenin, however the region that is contained between amino acids 65 and 72 in RNase is different in angiogenin. In fact, angiogenin lacks the disulfide bridge formed by the cysteines at positions 65 and 72 in RNase. (see Appendix II).

Harper and Vallee (1989) using regional mutagenesis were able to replace residues 58-70 of angiogenin by the corresponding segment of RNase (residues 59-73). Expression of this hybrid in *Escherichia coli* produced a hybrid protein that had a reduced angiogenicity but an increased enzymatic activity. With RNA and tRNA as substrates, the hybrid molecule is 660- and 300-fold more active than angiogenin respectively. The enzymatic activity against polyuridylic acid, polycytidylic acid, CpA and UpA is enhanced about 200-fold.

This data obtained by Harper and Vallee, (1989) is in accordance with the findings published in this thesis. In this work, amino acids which are in the region 65-72 of RNase have been substituted by alanine using site-directed mutagenesis. Some of the mutants thus produced have shown a decreased activity towards dinucleotide substrates (see Chapter 5).

The roles of His-13 (His-12 in RNase) and His-114 (His-119 in RNase) in the ribonucleolytic activity of angiogenin have been investigated by Shapiro and Vallee (1989) using site-directed mutagenesis. Replacement of either residue by alanine

(H13A and H114A) decreases enzymatic activity by at least 10000-fold and the angiogenic activity is virtually abolished. Mutations of these histidines to glutamine (H13Q) or Asn (H114N) produce derivatives which are less active than the native enzyme when using t-RNA or CpA as substrates.

Shapiro *et al.*, (1989) had also substituted Lys-40 (Lys-41 in RNase) by glutamine or arginine by site-directed mutagenesis. They found that these derivatives had a reduced angiogenic and enzymatic activities.

The fact that all these mutants have reduced angiogenic and enzymatic activities, strongly suggests that the formation of blood vessels could be related or is dependent on an intact active site.

The large reduction in enzymatic activity of the mutants H13A and H114A in angiogenin (Saphiro and Vallee, 1989) confirms almost definitely that two histidines are responsible for the mode of action of RNase as it was originally proposed by Findlay and collaborators (1961).

### **1.5.3 Bovine seminal ribonuclease (BS-RNase)**

The ribonuclease found in bull semen, although a member of the mammalian superfamily of RNases, possesses some unusual properties, including anti-spermatogenesis, antitumor activity and immunosuppression (Allesio *et al.*, 1991). These type of ribonucleases have been designated RISBASES (Ribonucleases with Special, i.e. non-catalytic, Biological Actions). Other members include angiogenin (see section 1.5.1), selective neurotoxic RNases like eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) which have some homology to pancreatic RNase and possess RNase as well as cytotoxic activities (Slifman *et al.*, 1986), and self-incompatibility factors from a flowering plant (McClure *et al.*, 1990).

BS-RNase is found "*in vivo*" as a dimer. Two inter-subunit disulfide bridges and non-covalent interactions are responsible for maintaining its dimeric structure. Each monomer shares 80% homology with pancreatic RNase (Allesio *et al.*, 1991).

BS-RNase contains two active sites; the key catalytic residues of each active site, His-12 and His-119, are contributed by the N-terminal tail of one chain and the main body of the other, respectively. The enzyme can degrade double and single stranded RNA, possibly due to the high positive net charge. It also has allosteric properties (Allesio *et al.*, 1991).

It certainly appears from the facts described up to now that evolution found the RNase structural scaffold, including the RNase active site, particularly convenient for the engineering of molecules which assist or interfere with key biological events.

### **1.5.3 Placental ribonuclease inhibitor**

However, nature has also provided a mechanism by which ribonuclease activity is tightly controlled. Human placental ribonuclease inhibitor (PRI) binds very tightly to angiogenin (0.71 fM) and to ribonuclease (44 fM) (Lee *et al.*, 1989). The amino acid sequence is composed of 460 amino acids with 7 internal repeat units of 57 amino acids each. These repeats are Leu-rich. PRI also contains 32 cysteine residues of which at least 30 are in the reduced form, it also contains a high number of acidic residues (Lee *et al.*, 1988).

Stopped-flow kinetic measurements carried out by Lee *et al.*, (1989) indicated a two-step mechanism for the binding of PRI to angiogenin. The first step involves rapid formation of an enzyme-inhibitor complex, followed by a slower isomerization to a much tighter enzyme-inhibitor complex. This inhibition would be of a competitive nature with the substrate. Thus, it could be possible that the subsites of RNase responsible for binding RNA are also responsible for binding the inhibitor.

Saphiro *et al.*, (1989) replaced the residue Lys-40 in angiogenin (equivalent to Lys-41 in RNase) by arginine using site-directed mutagenesis. The mutant produced decreases the binding affinity for PRI by 100-fold. The same authors replaced Lys-40 with Gln and the decrease in binding affinity for PRI was by a factor of 1300.

## 1.6 Microbial ribonucleases

Most of the microbial ribonucleases are specific for guanyl residues in the RNA molecule (see 1.6.1 and 1.6.2). Single stranded RNA is the preferred substrate for these enzymes and is cleaved in two steps as pancreatic ribonuclease.

Sevcik *et al* (1990) have studied the X-ray structures obtained from complexes of three guanyl-specific ribonucleases with guanosine-3'-monophosphate. From their studies, a binding site for the guanidylic residues was identified. This binding site comprised part of the polypeptide chain between residues 37 and 40 and the side chain of Glu-41.

### 1.6.1 Barnase

Barnase is a ribonuclease from *Bacillus amyloliquefaciens*, it is composed of a single polypeptide chain with no disulfide bonds. Mossakowska and collaborators (1989) have characterized the enzyme in terms of its basic kinetics and specificity. Using the technique of site-directed mutagenesis, they were able to identify the key residues involved in catalysis. In the light of these findings, it was proposed that Glu-73 is the general base and His-102 is the general acid in the cyclization reaction, the roles of these residues being reversed in the hydrolysis of the cyclic phosphate intermediate. Although barnase has a very low activity against dinucleotide (3'-5') substrates, it still shows a preference for the leaving group in substrates of the form GpN (where N is any base). Thus, the hydrolysis of dinucleotides of structure GpN, barnase prefers A > G > C > U at position N.

This low activity of barnase for the hydrolysis of dinucleotide substrates is in direct contrast to its efficient hydrolysis of RNA. Mossakowska and colleagues (1989) propose that the small substrates are binding non-productively and that further subsite interactions may be needed to be utilized to produce efficient hydrolysis. This indicates a cooperative effect, which is not found in pancreatic RNase (the hydrolysis of dinucleotide (3'-5') substrates is highly efficient, specially CpA). Perhaps evolution has perfected the ribonucleases found in mammals, so that the removal of unnecessary RNA from these highly organized systems is more efficient.

### 1.6.2 Ribonuclease T1 (RNase T1)

RNase T1 is a ribonuclease from *Aspergillus oryzae* and has been extensively studied. Steyaert and colleagues (1990) utilizing site-directed mutagenesis were able to elucidate the amino acids responsible for acid-base catalysis in RNase T1. The amino acid that acts as a general base is Glu-58 and the general acid is His-92. However, it was observed that upon replacement of Glu-58 by alanine, yielded an enzyme which still exhibits significant activity, and it was proposed that His-40 adopts the function of general base in derivatives of RNase T1 where Glu-58 is substituted by alanine.

The same authors (Steyaert *et al.*, 1991) indicated that for a series of dinucleotides of structure GpN, the enzyme behaves most efficiently on the following series of dinucleotide substrates GpC > GpA > GpG > GpU.

Studies by means of site-directed mutagenesis carried out by Steyaert *et al* (1992) indicate that RNase T1 contains a subsite which by interactions with the leaving nucleoside N of GpN substrates (where N can be any base), contributes to catalysis. The N36A and N98A mutations reduce the transesterification rate of GpC, GpA and GpU whereas they have virtually no effect on the transesterification kinetics of the synthetic substrate guanosine 3'-methyl phosphate (the leaving group N, is replaced by methanol). Furthermore, it was suggested that Asn-98 is at least in part responsible for the subsite preference for cytidine.

This data compares well with the work presented in this thesis. Substitutions of amino acids that are part of the B2 subsite in RNase yield mutant enzymes with a reduced catalytic efficiency towards dinucleotide substrates, but the hydrolysis of the cyclic phosphate intermediate is unaffected.

The amino acids Asn-36 and Asn-98 in RNase T1 are not conserved in the homologous family of microbial ribonucleases. In particular, Asn-98 of RNase T1 which is contained in a loop moiety, is absent in barnase (see 1.6.1). This could account for the poor activity of barnase towards dinucleotide (3'-5') substrates.

The difference between RNase T1 and barnase in the hydrolysis of dinucleotide substrates and their different specificities with respect to the leaving group is strikingly similar to the pair RNase-angiogenin, where angiogenin is a very poor catalyst for dinucleotide (3'-5') substrates (see 1.5.1) compared to the very and highly efficient pancreatic RNase.

The reason why microbial ribonucleases prefer a purine instead of a pyrimidine (as in pancreatic RNase) in the binding site of the enzyme is not known. The ratio  $k_{\text{cat}}/K_m$  exhibited by barnase for its best dinucleotide substrate is  $3600 \text{ M}^{-1} \text{ s}^{-1}$  (Mossakowska *et al.*, 1989). However, the  $k_{\text{cat}}/K_m$  exhibited by RNase T1 for its best dinucleotide substrate is between  $1600\text{-}2000 \text{ mM}^{-1} \text{ s}^{-1}$  (Steyaert *et al.*, 1991,1992). This is similar to the ratio  $k_{\text{cat}}/K_m$  that RNase exhibits for CpA,  $2700 \text{ mM}^{-1} \text{ s}^{-1}$  (see Chapter 5). The reasons for this differential specificity must be other than to produce a better catalyst.

## 1.7 Ribonuclease P (RNase P)

RNase-P is a ribozyme (an enzyme composed of RNA) that is involved in t-RNA biosynthesis. RNase-P catalyzes the endonucleolytic removal of 5' leader sequences from the precursor of t-RNA (pre-t-RNA), thus generating mature 5' ends of t-RNA. RNase-P or RNase-P-like activity has been found in bacteria, archaeobacteria, in

organelles like chloroplasts and mitochondria and in eukaryotes (Darr *et al.*, 1992 and references therein).

Although RNases have been extensively studied they exhibit kinetic characteristics that are not yet understood. In order to have a deeper knowledge of the specificity that RNase shows for different dinucleotide substrates (see table 1.1), it is necessary to produce genetically engineered mutations of the enzyme and assess their behaviour towards a range of substrates. Thus it becomes imperative to have an expression system which enables the production of sufficient recombinant RNase (and its mutants thereof) to carry out kinetic measurements and also a mutagenesis technique which allows the alteration of the coding sequence of the enzyme.

A description of the most used systems of expression in prokaryotes is presented, followed by an account of the different techniques used for introducing altered coding sequences into genes of interest.



## **1.8 Production of heterologous proteins in prokaryotes**

Expression or production of eukaryotic proteins in prokaryotes is greatly facilitated by the availability of plasmid DNA. Thus, a coding sequence of the protein of interest is inserted into a plasmid containing a promoter. This promoter contains sequences that facilitates the initiation of transcription of the DNA coding sequence of interest.

Thus, under certain conditions of bacterial growth, mRNA coding for the protein of interest is overproduced and the resultant protein product can be purified and characterized.

### **1.8.1 Properties of plasmid DNA**

Plasmids are circular extrachromosomal DNA molecules, found in most prokaryotes and also in some yeasts. Plasmids designed for recombinant DNA technology have to comply to certain requirements: they must be able to replicate autonomously in bacteria and they must confer upon the cells a new phenotype which will make it easy to differentiate it from cells which do not contain the plasmid. Plasmids become useful if they contain a whole spectrum of unique restriction endonuclease cleavage sites, thus making possible the insertion of foreign DNA with compatible ends. Plasmids should also be of low molecular weight, since the frequency of transformation into competent cells decreases with increasing molecular weight. Another important feature is that it may be desirable to maintain plasmids as multiple copies per cell, thus increasing the gene dosage that encodes a protein of interest (Winnacker, 1987).

### **1.8.2 Which is the ideal host for the expression of eukaryotic proteins?**

The answer very much depends on the structural and functional features of the protein to be produced. However, this simple question is crucial in designing a successful strategy for the production of heterologous eukaryotic proteins.

### **1.8.2.1 Production of heterologous proteins in *Bacillus subtilis*.**

An important advantage of using *B. subtilis* as a host for the expression studies is its ability to secrete proteins directly into the culture medium. This secretion is very useful since many proteins may be toxic to the cell if they remain in the cytoplasm. Also, many proteins require a specific environment for folding, thus the reducing conditions inside the cell do not allow for the folding of proteins containing disulfide bridges. Thus, secretion into the medium, where there is an oxidative environment, is the strategy of choice (Vasantha and Filpula, 1989).

However, an important disadvantage is that *B. subtilis* also naturally secretes proteases into the culture medium, which have the potential to degrade the protein of interest. Vasantha and Filpula, (1989) utilized this host for the production of recombinant bovine pancreatic RNase. The recombinant protein was found to be in the culture medium. However, proteolysis of the protein had occurred, resulting in lower yields than expected.

Two major naturally secreted proteases from *B. subtilis* have been identified and isolated by Stahl and Ferrari, (1984). This has enabled the production of strains which contain deletions of genes encoding for these two proteases. However, much work is still needed to eliminate all the proteases that *B. subtilis* naturally secretes. Another factor which can contribute to the instability of recombinant proteins in the culture medium is the agitation that is produced during fermentation. The resulting denaturation of the protein could certainly increase its susceptibility to proteases (Henner, 1990).

### **1.8.2.2 Production of eukaryotic proteins in *Escherichia coli*.**

*E. coli* has been extensively studied over the years and provides immense possibilities for the expression of heterologous proteins. Proteins produced in *E. coli* can be directed to three major locations. Proteins can be produced intracellularly, exported to the periplasm or even secreted into the medium. The choice of a particular strategy

depends mainly on the potential toxicity and stability that the protein of interest may attain in a given location of the cell. Also, the successful purification of a recombinant protein may be highly affected by where this protein is located. It is clear that proteins containing disulfide bridges will not be folded in the intracellular compartment of the cell. Thus directing production of recombinant protein into more oxidative environments like the periplasmic space of *E. coli* or secretion into the culture medium is required for such protein to be in a soluble state. The export of heterologous proteins to the periplasm, greatly facilitates its subsequent isolation. Procedures for selectively releasing the periplasmic contents have been extensively developed (C. French *et al.*,<sup>unpublished</sup> ). The overproduced protein is then easily purified by chromatographic procedures.

Secretion of heterologous proteins into the culture medium also facilitates their purification. However, the initial volume in which the protein of interest has to be purified from is very large compared to the periplasmic fraction (see chapter 3).

Intracellular expression is required if these two strategies (export to the periplasm and secretion to the culture medium) cannot be carried out because of the specific nature of the protein. Purification of heterologous proteins, produced intracellularly can be facilitated if they are overproduced and soluble. Proteins which form inclusion bodies present an additional problem in that these inclusion bodies have to be solubilized before chromatographic procedures are carried out.

## **1.9 Production of heterologous proteins in eukaryotes.**

A short section is required to briefly outline the two major systems of expression using eukaryotic hosts.

### **1.9.1 Utilization of yeast for production of heterologous proteins.**

The choice between yeast and *E. coli* depends on the inherent characteristics of the protein to be produced. For proteins which require post-translational modifications

such as glycosylation yeast is the organism of choice, since as a eukaryote it has the mechanisms required for the addition of sugars to proteins.

Raines,(1989) has successfully produced RNase using yeast as a host. In this system, the production of RNase was directed to the culture medium using a yeast signal sequence. The yields of RNase obtained were sufficient for its characterization.

### **1.9.2 Production of heterologous proteins using mammalian cell lines.**

The use of mammalian cells as hosts for the production of recombinant proteins is still in its early days. The inconvenience of these systems could be summarized as follows; techniques are expensive and the method for the introduction of DNA is via viral infection or direct injection. Problems are also associated in maintaining a stable cell line.

### **1.10 Design of plasmids for the production of recombinant proteins in *Escherichia coli*.**

The main properties that a plasmid must have are described in section 1.8.1. However, plasmids utilized for expression studies should also contain a promoter sequence near a multiple cloning site. This site, containing unique restriction endonuclease cleavage sites, is then utilized to insert the coding sequence for the protein of interest downstream of the promoter. In many cases, the activity of the promoter should be regulated since recombinant proteins may be toxic to the cell. For example the *tac* promoter, which has been utilized in this work to control the expression of RNase in *E. coli*, is normally repressed by cells containing the gene for the repressor molecule. Thus, under normal conditions of growth, the promoter remains almost inactive. However, upon addition of the inducer IPTG, the promoter is then activated and transcription of the coding sequence for RNase takes place.

McGeehan and Benner, (1989) have used the  $\lambda$  promoter to overproduce RNase intracellularly in the form of inclusion bodies in *E. coli*. The  $\lambda$  promoter is tightly

regulated under normal condition of growth (37 °C), however when the temperature of growth is raised to 42 °C , the promoter becomes derepressed and production of the protein starts. Nambiar *et al.*, (1987) have used the *lac* promoter (which is activated using the inducer IPTG), to express a  $\beta$ -galactosidase-RNase hybrid protein in *Escherichia coli*.

The above authors (McGeehan and Nambiar) have been able to produce recombinant RNase in *Escherichia coli*. However, the recombinant protein was found to be inside the cell forming inclusion bodies. Thus, prior to purification of the protein, the inclusion bodies had to be solubilized and refolding experiments were necessary to obtain an active enzyme. A solution to this problem could be to target the production of RNase into a more oxidative environment, like the periplasmic space of *E. coli*.

Plasmids containing prokaryotic signal sequences fused to promoters have been used for the export of recombinant proteins into the periplasmic space of *E. coli* (Hsiung *et al.*, 1986). Gray *et al.*, (1985) have also made a comparative study on the export of the human growth hormone (hGH) using a signal sequence derived from *E. coli* and the natural signal sequence of hGH. The results obtained are comparable in that both signal sequences are recognized by *E. coli* and translocate the protein into the periplasm. However, in other cases, this approach has been only partially successful, since the sequence of the mature protein also effects its ability to be exported to the periplasm (Schein, 1989).

Very recently, Schein *et al.*, (1992) have fused a synthetic gene coding for bovine pancreatic RNase to a signal sequence for the murine spleen ribonuclease. The recombinant pancreatic RNase produced was found to be in the periplasm of *E. coli* and also in the culture medium.

In some cases, proteins that are designed to be exported to the periplasm also appear in the culture medium (Schein *et al.*, 1992). This passive secretion takes place not by

design but rather by default. This is probably due to damage to the cell envelope caused by high levels of expression over prolonged periods of time, thus resulting in the protein leaking into the medium. However, it is possible to engineer a system in which the protein produced is specifically secreted into the medium. Kato *et al.*, (1987) co-produced two proteins in *E. coli*, one was the protein of interest and the other a protein which permeabilizes the outer membrane of *E. coli*. In this system, both proteins are exported to the periplasm and the subsequent release of the protein of interest is facilitated by the other, which permeabilizes the outer membrane.

The strategy for the successful expression of RNase presented in this thesis is as follows: the host used to produce recombinant bovine pancreatic RNase has been *Escherichia coli*. Since RNase does not contain sugars, expression in yeast is not necessary. The entire coding sequence for the RNase precursor has been inserted downstream of a regulated promoter (*tac* promoter) and its expression has been directed to the periplasmic space of *E. coli* by virtue of the natural signal sequence of pre-RNase (see Chapter 3).

### **1.11 Purification of ribonucleases.**

The method for purifying bovine pancreatic ribonuclease from a minced pancreas involves the use of cation-exchange chromatography. Hirs *et al.*, (1953) used the carboxyl ion exchange resin IRC-50 to purify RNase. Taborsky (1959) utilized a system of purification based on carboxymethyl cellulose and Crestfield *et al.*, (1963) found sulfoethyl Sephadex very useful.

The strategy for purification of recombinant RNase from the periplasmic contents released after a spheroplastic/osmotic shock procedure (C. French *et al.*, unpublished) has also involved the use of cation-exchange chromatography (see Chapter 3). However, the use of a compatible FPLC column (Mono-S 5/5) which contains sulfonic acid groups facilitates the purification of RNase to homogeneity since the resolving capacity of this column is superior to the conventional resins (see Fig. 3.6).

## **1.12 Available techniques for altering the DNA sequence coding for a specific protein.**

Since the early days in which genetic mutations were achieved by irradiation with ultraviolet light, much improvement has been achieved in this field. The discovery of restriction enzymes, single stranded DNA vectors and the thermostable enzyme *taq* polymerase has allowed for the development of techniques for the site-directed mutation of a coding sequence in any given gene.

Site-directed mutagenesis thus enables us to increase our knowledge of the relationship between structure and function of a given protein. In this thesis, genetically engineered mutants of RNase have been produced (see Chapter 4) in order to provide a deeper understanding of the diverse specificity that RNase shows for dinucleotide substrates (see Chapter 5).

### **1.12.1 Site-directed mutagenesis using single stranded DNA vectors.**

Zoller and Smith, (1982) have made oligodeoxyribonucleotide directed site-specific mutations in DNA fragments cloned in M13 derived vectors or plasmids. The oligonucleotide carrying an altered sequence is hybridized to single stranded DNA. The annealed oligonucleotide-template hybrid is then extended using the Klenow fragment and ligated using T4 DNA ligase. The resulting double stranded circular DNA is then transformed into competent cells of *E. coli*. Single stranded DNA, obtained from individual plaques, containing a copy of the gene with a coding sequence specifically altered is used for sequencing to verify the incorporation of the mutation.

## **1.12.2 Use of the polymerase chain reaction (PCR) for site-directed mutagenesis.**

### **1.12.2.1 The overlap extension technique.**

The overlap extension method represents an approach whereby complementary oligodeoxyribonucleotides (or primers) and the polymerase chain reaction are used to generate two DNA fragments having overlapping ends. These fragments are then denatured and annealed allowing the 3' overlap of each strand to serve as a primer in a further PCR incubation. The double stranded DNA fragment produced containing the altered sequence needs to be inserted into an appropriate plasmid to verify the sequence. Ho *et al.*, (1989) have utilized this technique to carry out site-directed mutagenesis on the major histocompatibility complex class-I gene.

### **1.12.2.2 The recombinant circle polymerase chain reaction (RCPCR). An improved technique for site-directed mutagenesis using PCR.**

The overlap extension technique using PCR as a means of introducing altered coding sequences in a specific gene has the drawback that the blunt ended fragment produced has to be inserted into an appropriate vector.

RCPCR (Jones and Howard, 1990) is an improved technique which eliminates this problem, and incorporates mutations in a DNA of interest with very few manipulations (as compared to the use of single stranded DNA vectors). The RCPCR technique involves two separate PCR incubations. In each of them, one of the two primers used contains the altered coding sequence. Plasmid DNA with the coding sequence of interest is used as template. The double stranded DNA fragments which are produced after the two PCR incubations are combined, denatured and re-annealed. The resulting products have discrete cohesive single stranded ends in addition to the original blunt ended products. These cohesive ends which are produced by the specific design of the primers used in each PCR incubation, allow for the formation of circles of DNA. These circles are used to directly transform competent cells (see Fig. 2.1 in Chapter 2).



This section has outlined the possible hosts for the expression of RNase, and some available techniques for site-directed mutagenesis have been described. The study presented in this thesis requires the production of recombinant bovine pancreatic RNase and its genetically engineered mutants produced using the RCPCR technique (see Chapter 4), in sufficient quantities for their kinetic characterization using dinucleotide substrates (see Chapter 5). Therefore, *Escherichia coli* has been the host of choice since it has been possible to utilize the natural signal sequence of RNase to export the recombinant protein to the periplasmic space, thus facilitating enormously its purification and isolation (see Chapter 3).

This combination of strategies has produced a system which is fast, highly efficient and allows for the rapid assessment of the effects that a specific RNase mutant has on the hydrolysis of dinucleotide substrates.

## CHAPTER 2: MATERIALS AND METHODS

All chemicals used in the experiments described in this thesis were supplied by BDH, Fisons or Sigma (unless indicated). They were of AnalaR grade or of the highest grade available.

### 2.1 Bacterial strains and commercial plasmids.

The *E. coli* strain used as a plasmid host and for expression studies was JM107, which is characterized by the genotype; *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*,  $\Delta$  (*lac-pro AB*), (Yanisch-Perron et al., 1985).

### 2.2 Culture medium and antibiotics.

Growth of *E. coli* JM107 under standard conditions was carried out at 37 °C, however for expression studies, growth was carried out at 28 °C. The medium used was nutrient broth (Oxoid) or nutrient agar (Oxoid nutrient broth solidified with 2% w/v Bacto agar). For expression studies, a medium was used which contained  $\text{KH}_2\text{PO}_4$  (2.3 g/l),  $\text{K}_2\text{HPO}_4$  (3.78 g/l), b actotryptone (12 gr/l), yeast extract (24 gr/l) and 0.4% (v/v) of glycerol (Tartoff and Hobbs, 1987). *E. coli* JM107 was also grown on minimal medium plates (salts, glucose and vitamin B1 or thiamine, see 2.4).

These medium were supplemented (if required) with ampicillin (500  $\mu\text{g/ml}$  for solid medium or 100  $\mu\text{g/ml}$  for liquid medium), IPTG (40  $\mu\text{g/ml}$ ) and X-Gal (80  $\mu\text{g/ml}$ ).

### 2.3 Maintenance of bacterial species.

*E. coli* JM107 was maintained on nutrient agar plates at 4 °C or in cell suspensions containing 20% (v/v) glycerol at -70 °C if longer storage was required.

#### **2.4 Preparation of competent cells.**

An *E. coli* JM107 colony was taken from a minimal medium plate and used to inoculate 5 ml of nutrient broth. After growth at 37 °C overnight with shaking, an aliquot (1 ml) was used to inoculate 200 ml of nutrient broth. Growth was carried out for 2-3 hours at 37 °C with shaking. The cells were pelleted by centrifugation at 8300 x g (ave) for 10 min. at 4 °C. The pellet was resuspended in 40 ml of ice cold 75 mM CaCl<sub>2</sub>, 15% (v/v) glycerol. The suspension was re-centrifuged as before and the pellet resuspended in 5 ml of ice cold 75 mM CaCl<sub>2</sub>, 15% (v/v) glycerol. The final suspension was stored in 0.5 ml aliquots, which were dispensed into pre-chilled sterile, glass bijoux bottles or pre-chilled sterile Falcon tubes. The aliquots were stored at -70 °C.

#### **2.5 Transformation with plasmid DNA using competent cells.**

To transform plasmid DNA into competent cells, an aliquot of competent cells (see 2.4) was mixed with plasmid DNA and left on ice for 45 minutes. This mixture was incubated for 10 minutes at 37 °C. The transformed cells were poured into 5 ml of nutrient broth and grown for 2-3 hours at 37 °C with shaking. An aliquot of the transformation mixture (100 µl) was spread onto an appropriate selective agar plate.

#### **2.6 Phenol extraction.**

For some procedures described in this thesis such as sequencing (see 2.16) and PCR (see 2.13), the DNA used is required to be substantially free of protein. This is achieved using phenol/chloroform extraction.

Solutions containing DNA for extraction were mixed with an equal volume of phenol/chloroform (50:50 v/v). The solution was vortexed for 2 sec. and centrifuged at 10000 x g (ave) for 10 min. at room temperature. The aqueous phase containing extracted DNA was removed and stored at 4 °C or used immediately.

The phenol used was treated as follows; 500 g of phenol were resuspended in 200 ml of water, 30 ml of 1 M Tris/HCl pH 7.4, 15 ml of 2 M NaOH and 60 ml of 0.2 M Na<sub>2</sub>EDTA.

## **2.7 Ethanol precipitation.**

To precipitate DNA from solution, 1/10 volume of 4 M CH<sub>3</sub>COONa or 5 M NaCl was added followed by 2 volumes of ice cold ethanol (no salt was added when precipitating RNase, see 3.2.3). After vortexing the suspension was placed at -20 °C for 20 minutes. Precipitated DNA was then pelleted by centrifugation at 10000 x g (ave) for 10 min. at room temperature. The supernatant was discarded. The pellet was washed with 1 ml of ice cold ethanol by centrifugation as above for 1 min. The pellet obtained was dried at 50 °C. Sterile water or TE buffer (10 mM Tris/HCl pH 7.5, 1 mM Na<sub>2</sub>EDTA, 20 µg/ml ribonuclease A (optional)) was used to resuspend the dried DNA (20-500 µl) which was stored at -20 °C.

## **2.8 Plasmid isolation from *E. coli*:**

### **2.8.1 Small scale (mini-prep).**

Small scale preparation of plasmid DNA was carried out as described by Birnboim and Doly (1979). An aliquot (1.5 ml) was removed from a 5 ml overnight culture of *E. coli* bearing the plasmid of interest. Cells were pelleted by centrifugation at 10000 x g (ave) for 5 min. at room temperature. The pellet was resuspended in 100 µl of lysis buffer (0.9% (w/v) glucose, 50 mM Tris/HCl pH 7.5, 1 mM Na<sub>2</sub>EDTA), and lysis was completed by the addition of 200 µl of 0.2 M NaOH, 1% (w/v) SDS. This mixture was inverted several times. To neutralize the solution, 150 µl of 4 M CH<sub>3</sub>COONa pH 5.1 was added and the suspension was vortexed thoroughly followed by an incubation on ice for 15 minutes. Cell membranes and debris were removed by centrifugation at 10000 x g (ave) for 5 min. at room temperature and the supernatant was transferred to a sterile tube. Phenol extraction (see 2.6) and ethanol precipitation (see 2.7) were performed to extract plasmid DNA from contaminant proteins, and a

pellet was obtained which was dried and resuspended in 100  $\mu$ l of sterile water or TE buffer.

When preparing plasmid DNA for double stranded DNA sequencing, ribonuclease (20  $\mu$ g/ml) was added after the phenol extraction step and an incubation was carried out at 37 °C for 30 minutes. Plasmid DNA was then phenol extracted and ethanol precipitated. The dried pellet was usually resuspended in 25  $\mu$ l of sterile water.

### **2.8.2 Large scale.**

Large scale preparation of plasmid DNA from *E. coli* was performed as described by Birnboim and Doly (1979). Cells from an overnight culture (400 ml) were pelleted by centrifugation at 8300 x g (ave) for 10 min. at 4 °C. The cells were resuspended in 20 ml of lysis buffer. This was followed by the addition of 40 ml of 0.2 M NaOH, 1% (w/v) SDS to complete lysis. To neutralize the solution, 50 ml of 4 M CH<sub>3</sub>COONa pH 5.1 was added and the suspension was mixed thoroughly prior to incubation on ice for 15 minutes. Cell membranes and debris were removed by centrifugation at 8300 x g (ave) for 30 min. at 10 °C. The supernatant was transferred to a clean tube. The plasmid DNA was precipitated, along with other contaminants, by adding 50 ml of 50% (w/v) polyethyleneglycol 6000 (PEG 6000) and mixing thoroughly. The precipitation of plasmid DNA was facilitated by incubation on ice for at least 1 hour. Precipitated plasmid DNA was pelleted by centrifugation at 2500 x g (ave) for 15 min. at 10 °C. The pellet was resuspended in 5 ml of TE buffer and the plasmid DNA was phenol extracted. The aqueous phase was removed and adjusted to a volume of 5 ml with TE buffer. CsCl was added to give a density of 1.1 g/ml and ethidium bromide was added to a final concentration of 0.6 mg/ml. The mixture was then centrifuged at 163000 x g (ave) for 16 hours at 20 °C. To increase resolution of bands, the speed of centrifugation was reduced to 104000 x g (ave) for the last 30 minutes. The band containing plasmid DNA was visualized with an ultraviolet lamp and removed with a syringe.

### **2.8.2.1 Extraction of intercalating ethidium bromide from plasmid DNA.**

The plasmid DNA solution was extracted three times with a saturated solution of CsCl/isopropanol to remove intercalating ethidium bromide. For each extraction, an equal volume of the saturated solution was mixed with the DNA. It was then thoroughly mixed and left on the bench until phase separation occurred. The upper layer was discarded. The volume of the bottom layer was measured and TE buffer was added to a final volume of 5 or 10 ml. Phenol extraction and ethanol precipitation (see 2.6 and 2.7) was then carried out. The dried pellet was resuspended in 250-500  $\mu$ l of sterile water or TE buffer.

### **2.9 Preparation of agarose gels**

DNA fragments from restriction digests (see 2.10) and PCR (see 2.13) were separated using agarose gel electrophoresis. The concentration of agarose was 1% (w/v) when resolution of high molecular weight DNA was required or 2% (w/v) when resolution of low molecular weight DNA was required.

Gels were prepared on 20 x 20 cm glass plates. Autoclave tape was applied around the edges of the plate to cast a mould. The agarose solution (either 1 or 2% w/v) was prepared by heating 1 or 2 g of high melting temperature agarose in 100 ml of electrophoresis buffer (90 mM Tris/base, 90 mM boric acid, 10 mM Na<sub>2</sub>EDTA and 0.5  $\mu$ g of ethidium bromide).

A 14 or 20 well comb was positioned at the top of the glass plate and the agarose solution poured into the mould at a temperature of 50 °C. Before transferring the agarose gel to the electrophoresis tank, the autoclave tape was removed. The separation of the DNA bands was performed by horizontal electrophoresis for 2 hours at 200 volts in electrophoresis buffer.

### **2.10 Restriction enzyme digestion.**

The appropriate restriction enzyme (10 units, 5 units/ $\mu$ l) was mixed with 2  $\mu$ l of 10 x restriction buffer (500 mM Tris/HCl pH 7.5, 50 mM MgCl<sub>2</sub> and NaCl; the concentration of salt varies with the restriction enzyme used) and plasmid DNA (1-5  $\mu$ g). The volume of the reaction mixture was adjusted to 20  $\mu$ l with TE buffer and the digestion was performed for 2-3 hours at 37 °C. The reaction was terminated by the addition of 5  $\mu$ l of 5 x stop mix (0.1 M Na<sub>2</sub>EDTA, 40% (w/v) sucrose, 0.15 mg/ml bromophenol blue).

The mixture was loaded onto an agarose gel and electrophoresed at 200 volts for 2 hours. DNA bands were visualized on a UV transilluminator and photographed if necessary.

### **2.11 Dephosphorylation of plasmid DNA.**

Digested plasmid DNA was dephosphorylated by the addition of 2 units (1 unit/ $\mu$ l) of calf intestine alkaline phosphatase (CIP). The mixture was then incubated for 30 min. at 37 °C. The plasmid DNA was phenol extracted (see 2.6) and stored at -20 °C.

### **2.12 Ligations.**

Ligation reactions were performed with the DNA to be ligated, T4 DNA ligase (20 units, 10 units/ $\mu$ l) and 1/10 volume of 10 x ligase cocktail (600 mM Tris/HCl pH 7.5, 100 mM MgCl<sub>2</sub>, 100 mM dithiothreitol, 1 mM ATP). The volume of the sample was adjusted to 20-30  $\mu$ l with TE buffer. The ligation reaction was left to proceed overnight at room temperature.

### **2.13 Polymerase Chain Reaction (PCR).**

To obtain an expressible coding sequence for RNase precursor from pQR138 (see 2.13.6) (see Fig. 3.1A), primers were synthesized complementary to the 5' and 3' ends of the coding sequence for RNase precursor and used in a PCR incubation.

### **2.13.1 Design of primers for PCR.**

The primers were designed to allow for the addition of new sequences at each end of the coding sequence for bovine pancreatic pre-ribonuclease A (see Fig. 3.1B).

A primer, designated primer 10, was synthesized which contains the coding sequence for the first 16 nucleotides of pre-ribonuclease. This primer contains an additional sequence encoding 6 amino acids which contains an initiation codon, a ribosome binding site (RBS) and a termination codon which overlaps the initiation codon of pre-ribonuclease. Primer 10 also contains an *EcoRI* restriction site with four additional bases to ensure correct cleavage (see 2.15).

A second primer, designated primer 11, was synthesized and contains the termination codon for RNase, an *EcoRI* site and 4 additional bases to ensure correct cleavage.

The DNA fragment that results from a PCR incubation using these two primers, will have 2 sets of coding sequences. One set contains the coding sequence for a hexapeptide and the other contains the coding sequence for RNase precursor. This PCR fragment will be cloned into an expression vector (see 2.18). The transcription of this fragment produces a bicistronic mRNA which upon translation produces a hexapeptide and the RNase precursor. Therefore the DNA fragment obtained after a PCR assay using primers 10 and 11 has been designated “2-cistron fragment” (see 2.13.6).

### **2.13.2 Synthesis of primers.**

The primers were synthesized by the phosphite-triester method, using cyanoethyl phosphoramidites, in a Cyclone DNA synthesizer (Milligen/Millipore).

### **2.13.3 Elution and deprotection of primers.**

The primer was eluted the column in which it was synthesized using concentrated ammonia. To remove cyanoethyl groups protecting the phosphate, 1 ml polypropylene



syringes were applied at each end of the column, and ammonia was passed through the column back and forwards several times. The column was left filled with ammonia at room temperature for 45 min. This procedure was repeated. The primer eluted from the column in concentrated ammonia was incubated at 65 °C for 5 hours (or at 55 °C overnight). This treatment liberates the primer from benzoyl groups protecting the amide in adenines and cytosines and isobutyryl groups protecting the amide in guanines. Water (3 volumes) was added to dilute the ammonia prior to freeze drying the sample. The lyophilizate was resuspended in 500  $\mu$ l of TE buffer and passed through a column of DNA grade sephadex G-25 (NAP-5, Pharmacia). The primer was eluted in 1 ml of TE buffer or sterile water.

#### **2.13.4 Purification of primers.**

Eluted primer contains not only the primer synthesized but also non-terminated chains that can compete with the full length primer in PCR experiments. To facilitate separation of other sequences from full length primer, a combination of anion exchange and reverse phase chromatography was carried out using Fast Protein Liquid Chromatography (FPLC, Pharmacia-LKB). This procedure is recommended for primers that are 30-75 bases long. For primers that are less than 30 bases, anion exchange chromatography should be sufficient.

##### **2.13.4.1 Anion-exchange chromatography.**

The column used was a Mono-Q 5/5 (Pharmacia-LKB) and elution was carried out using buffer A, 10 mM NaOH pH 12, 0.5 M NaCl and buffer B, 10 mM NaOH pH 12, 0.9 M NaCl. A gradient was applied from 0-100% B in 30 minutes at a flow rate of 1 ml/min. Fractions (1.0 ml) were collected. The major peak was collected. For primers <30 bp in length, the collected peak was desalted on a NAP-5 column. For primers >30 bp in length, the desalting step was unnecessary since further purification was to be carried out by reverse phase chromatography (see 2.13.4.2).

#### **2.13.4.2 Reverse phase chromatography.**

The column used was a ProRPC-HR 5/10 (Pharmacia-LKB). The pH of the collected peak (see 2.13.4.1) was adjusted to 7.0 with acetic acid. A fraction of the collected peak (20-30%) was applied to the column and elution was carried out using buffer A, 100 mM TEAA pH 7.0, 10% (v/v) acetonitrile and buffer B, 100 mM TEAA pH 7.0, 20% (v/v) acetonitrile. A gradient was applied from 0-100% B in 30 minutes at a flow rate of 1 ml/min. Fractions (1.0 ml) were collected. The major peak was collected and rotary evaporated at 50 °C. Sterile water (100 µl) was added to wash the precipitate, and after a final evaporation, the dried material was resuspended in 500 µl of sterile water.

#### **2.13.5 Determination of primer concentration.**

The concentration of primers was determined by their base composition using the millimolar extinction coefficients for each base of ( $\epsilon_{(A)}=15.4$ ,  $\epsilon_{(C)}=9.2$ ,  $\epsilon_{(T)}=9.7$ ,  $\epsilon_{(G)}=11.4$ ). The concentration of a given primer can be calculated according to the equation:

$$[\text{primer}] = \text{OD}_{260}(\text{per ml}) / \epsilon = X \text{ mM}$$

Where  $\epsilon$  represents the sum of the extinction coefficients attributable to the total number of As, Cs, Ts and Gs for a given primer.

#### **2.13.6 Production of a 2-cistron fragment using PCR.**

The plasmid pQR138 was used as a template in all PCR incubations for the production of 2-cistron fragments (see 2.13.1). pQR138 is a plasmid chimaera comprising pBR322 and a 1.14 Kb fragment containing a cDNA of 450 bp which codes for RNase precursor and was kindly supplied by Prof. A. Furia (Italy) (Carsana *et al.*, 1988).

PCR incubations contained template (100 ng), primers 10 and 11 (50 pmols each) (see 2.13.1), nucleotides (0.2 mM), 20 mM Tris/HCl pH 8.0, 15 mM  $(\text{NH}_4)_2\text{SO}_4$ , 2 mM  $\text{Mg}_2\text{Cl}$ , 0.05% NP40 and 0.05% Tween 20 in a total volume of 50 or 100 µl. The

reaction components were rapidly mixed followed by centrifugation at 10000 x g (ave) for 2 seconds at room temperature. An equal volume of paraffin oil was added to avoid evaporation during the reaction. Template DNA was fully denatured by incubation for 5 min. at 92 °C. and the reaction was initiated by the addition of Tet/Z polymerase (2 units, 1 unit/ $\mu$ l). The reaction was allowed to undergo 25 cycles in a thermal cycler (Techne 2000). See section 3.1.2 for conditions of the PCR incubation.

At the end of the reaction, an aliquot (10%) was removed and mixed with 2.5  $\mu$ l of stop mix (see 2.10). This was loaded onto a 1.6% agarose gel and electrophoresed for 2 hours at 200 volts.

#### **2.14 Treatment of the 2-cistron fragment prior to restriction enzyme digestion.**

The 2-cistron fragment generated using PCR (see 2.13.6) contains *EcoRI* sites at each end. For these sites to be cleaved efficiently it is important that the fragment has blunt or flushed ends.

DNA polymerase I (Klenow fragment) (20 units, 10 units/ $\mu$ l) was added to the completed PCR assay and an incubation was carried out for 30 min. at 37 °C. Phenol extraction and ethanol precipitation (see sections 2.6 and 2.7) was carried out. The resultant pellet was resuspended in 20  $\mu$ l of TE buffer.

#### **2.15 Ligation of the 2-cistron fragment into pUC18. Production of pQR162.**

The plasmid pUC18 contains the  $\beta$ -galactosidase gene under the control of the *lac* promoter. This gene is normally repressed, but when inducer (IPTG) is added to the medium, the production of  $\beta$ -galactosidase starts. *E. coli* cells producing  $\beta$ -galactosidase are clearly discernible from those not producing the enzyme when X-Gal is present in the medium. The hydrolysis of X-Gal will produce a blue colour which will stain the colony.

pUC18 also contains a series of unique restriction sites in the coding sequence of the  $\beta$ -galactosidase gene that can be used for insertion of foreign DNA. Thus, when ligation has been successful, the reading frame of the  $\beta$ -galactosidase gene is disrupted and white colonies will appear on agar plates containing ampicillin, IPTG and X-Gal (see 2.2).

pUC 18 (5  $\mu$ g) was digested with *EcoRI* (see 2.10). Incubation for 10 min. at 70 °C was carried out to inactivate the restriction enzyme. The cleaved plasmid was dephosphorylated (see 2.11). Phenol extraction was carried out (see 2.6) to denature the alkaline phosphatase. The 2-cistron fragment treated as in section 2.14 was digested with *EcoRI* for 3-4 hours at 37 °C. The restriction enzyme was then heat inactivated as above.

Digested and dephosphorylated pUC18 (1  $\mu$ g) was mixed with the cleaved 2-cistron fragment (200 ng) and ligation was carried out overnight at room temperature in the presence of T4 DNA ligase (see 2.12). The ligated DNA was added to an aliquot of competent cells (see 2.4) and its transformation was carried out (see 2.5). An aliquot (100  $\mu$ l) of the transformation mixture was spread onto agar plates that contained ampicillin, IPTG and X-Gal (see 2.2) and grown overnight at 37 °C.

A mixture of blue and white colonies were obtained. Single white colonies were selected and streaked on agar plates as described above, and grown in 5 ml of nutrient broth. Mini-preps were carried out (see 2.8.1) to isolate the plasmid. The plasmid DNA was digested with *EcoRI* to reveal the size of the insert (see results). The plasmid which contained the 2-cistron fragment insert (approximately 500 bp) was designated pQR162.

The clone harbouring pQR162 was grown in 400 ml of nutrient broth. Large scale isolation of pQR162 using CsCl gradients was carried out (see 2.8.2) in order to have

sufficient DNA for double stranded DNA sequencing and for subcloning the 2-cistron fragment into plasmid pKK223.3 for expression studies.

### **2.16 Double stranded DNA sequencing.**

Plasmid DNA (2.5  $\mu\text{g}$ ) purified by CsCl gradient centrifugation (see 2.8.2) or plasmid DNA (4-5  $\mu\text{g}$ ) obtained after mini-prep procedures (see 2.8.1) was used as a template for double stranded DNA sequencing.

#### **2.16.1 Treatment of glass plates.**

The glass plates used for sequencing had dimensions of 41.8 cm x 33.5 cm x 5 mm and 39.4 cm x 33.5 cm x 5 mm and had bevelled edges. They were treated before use with acetone and ethanol, and one side was siliconized (to facilitate the separation of the glass plates once electrophoresis had finished, see below). Adhesive tape was used to seal the plates together separated by 0.4 mm spacers.

#### **2.16.2 Preparation of polyacrylamide gels.**

Polyacrylamide gels (8%) (Laemmli *et al.*, 1970) were prepared by dissolving polyacrylamide (5.7 g), bis-acrylamide (0.3 g) and urea (36 g) in TBE buffer (90 mM Tris base, 90 mM boric acid and 10 mM  $\text{Na}_2\text{EDTA}$ ). The volume of the mixture was adjusted to 75 ml with TBE buffer. Ammonium persulfate 10% (w/v) (450  $\mu\text{l}$ ) and TEMED (50  $\mu\text{l}$ ) were then added to the mixture.

The glass plates were placed at an angle of  $45^\circ$  and the polyacrylamide solution was poured continuously to avoid air bubbles. The plates were then lowered to an angle of  $5^\circ$ . The flat side of two 48-well combs (0.4 mm width) were introduced 3 mm deep into the gel. Bulldog clips were applied to clamp the top of the plates. The polyacrylamide solution was left to polymerize for 30 min.

Once the polyacrylamide solution had polymerized, the bulldog clips, combs and tape were removed and the top of the gel was washed with distilled water. The sequencing

gel was then placed in a gel electrophoresis apparatus (Bethesda Research Laboratories Life Technologies Inc., Model S2) and TBE buffer was added to the top and bottom reservoirs. The surface left by the spacers was washed to remove excess polyacrylamide using a Pasteur pipette. The gel comb was then introduced until the teeth reached the polyacrylamide gel.

### **2.16.3 Electrophoresis**

Samples to be electrophoresed containing bromophenol blue and xylene cyanol (see 2.16) were incubated for 2-3 min. at 92 °C and placed immediately on ice prior loading the gel. Electrophoresis was carried out at 40 mA constant current. To obtain the maximum amount of sequence from one electrophoresis run, the gel was loaded three times with the same sequencing mixes. This was achieved by re-loading once the bromophenol blue dye marker had reached the bottom of the gel.

### **2.16.4 Treatment of the polyacrylamide gel after electrophoresis.**

On completion of electrophoresis (see 2.16.3), the plates were separated. The gel was fixed by soaking in a solution of methanol and acetic acid (5% v/v) for 30 min. The solution was aspirated and the gel washed for 5 min. with distilled water. The sequencing gel was removed from the plate by carefully transferring to a sheet of Whatman 3 MM and sealed with Saran wrap prior to drying. Drying was for 1 hour at 60 °C under vacuum.

Bands were visualized by autoradiography overnight at room temperature using Fuji X-ray film. The X-ray was developed using a commercial developer (X-OGRAPH Ltd., Model X2).

### **2.16.5 Sequencing reactions.**

Double stranded DNA sequencing was carried out by the dideoxy method of Sanger *et al.*, (1977) using a commercial T<sup>7</sup> Sequencing kit (Pharmacia-LKB).

Plasmid DNA (8  $\mu$ l) was denatured by adding 2  $\mu$ l of 2 M NaOH. The mixture was incubated for 10 min. at room temperature. The denatured plasmid DNA was precipitated by adding 3 M  $\text{CH}_3\text{COONa}$  (3  $\mu$ l), sterile water (7  $\mu$ l) and ice-cold absolute alcohol (60  $\mu$ l). The precipitation was carried out for 30 min. at  $-20^\circ\text{C}$ . Precipitated DNA was then pelleted by centrifugation at 10000 x g (ave) for 10 min. at room temperature. The pellet was washed with 200  $\mu$ l of ice-cold absolute alcohol by centrifugation at 10000 x g (ave) for 2 min. at room temperature followed by drying for 30 min. at  $55^\circ\text{C}$ . The denatured DNA was resuspended in 10  $\mu$ l of sterile water.

Annealing was initiated by the addition of primer (2  $\mu$ l) and annealing buffer (2  $\mu$ l) and was carried out for 20 min at  $37^\circ\text{C}$ . The annealed product was then left to cool for at least 10 min. at room temperature.

An aliquot (2.5  $\mu$ l) of A mix, C mix, T mix and G mix was dispensed in 4 sterile tubes.

T7 DNA polymerase stock (7 u/ $\mu$ l) was diluted to 1.5 u/ $\mu$ l with enzyme dilution buffer and placed on ice until needed. An aliquot of the diluted enzyme (3 units) was used to carry out the termination reactions. An "enzyme premix" was prepared by adding in a microfuge tube (on ice), water (1 $\mu$ l), labelling mix (3  $\mu$ l), diluted T7 DNA polymerase (2  $\mu$ l) and  $^{35}\text{S}$ -ATP (1  $\mu$ l, 10  $\mu\text{Ci}/\mu$ l). The mixture was gently mixed and left on ice.

Labelling reactions were carried out by mixing 6  $\mu$ l of the "enzyme premix" with the annealed template and primer. The reaction was performed for 5 min. at room temperature. While the labelling reactions were in progress, the four sequencing mixes (A, C, T and G mix) were pre-warmed at  $37^\circ\text{C}$ . An aliquot of the labelling reaction (4.5  $\mu$ l) was transferred into each of the four prewarmed sequencing mixes (a fresh Gilson tip was used for each transfer) and the termination reactions were carried

out for 5 min. at 37 °C. Stop mix (5  $\mu$ l) containing bromophenol blue and xylene cyanol was added to each reaction and placed on ice prior to loading the gel (see 2.16.3) or stored at -20 °C.

### **2.17 DNA extraction from agarose gels.**

To obtain fragments generated by PCR or restriction enzyme digestion, agarose gel electrophoresis was carried out. Molecular weight markers generated by digestion of Lambda DNA with *Pst*I were electrophoresed alongside to determine the size of the resolved DNA bands.

Electrophoresis was carried out for 2 hours at 180 volts and the DNA bands were visualized on an UV transilluminator. A sharp razor was used to cut the agarose gel around the DNA band of interest. The gel fragment containing the DNA was introduced into a centrifugal filter unit (Spin-X, Costar) and centrifuged at 10000 x g (ave) for 10 min. at room temperature. Sterile water (100-200  $\mu$ l) was added to the unit and placed for 15 min. at -20 °C. The agarose was disrupted further, by incubation for 5 min. at 37 °C. The DNA was completely eluted from the agarose by a centrifugation as described above. The volume of the eluent was measured and ethanol precipitation was carried out (see 2.7). The dried pellet was resuspended in 25  $\mu$ l of sterile water.

### **2.18 Subcloning of the 2-cistron fragment into an expression vector (pKK223.3).**

The plasmid pQR162 (see Fig. 3.1A; pUC18 containing the 2-cistron fragment, see 2.15) was digested with *Eco*RI (see 2.10). The products of the digestion were electrophoresed and the DNA band of interest ( the 2-cistron fragment, approximately 500 bp) was gel extracted (see 2.17). Plasmid pKK223.3 was digested with *Eco*RI followed by dephosphorylation (see 2.11).

Digested and dephosphorylated pKK223.3 (3  $\mu$ l, approximately 1  $\mu$ g) was mixed with an aliquot of the 2-cistron fragment DNA (5  $\mu$ l, approximately 200 ng) and ligation



(see 2.12) was allowed to proceed overnight at room temperature. The products of the ligation were used to transform an aliquot of competent cells (see 2.4 and 2.5). The transformed cells were used to inoculate 5 ml of nutrient broth and grown for 3 hours at 37 °C with shaking. An aliquot (100  $\mu$ l) was spread onto agar plates containing ampicillin (see 2.2). Growth of the recombinants (*E. coli* cells which have taken up ligated DNA) was allowed to proceed overnight at 37 °C. No direct selection method is available for these recombinants as described in 2.15, therefore random colonies were selected and further grown overnight at 37 °C in agar plates containing ampicillin and in 5 ml of nutrient broth.

Mini-preps (see 2.8.1) were carried out on these recombinants to isolate the plasmid. Plasmid DNA from each recombinant was digested (see 2.10) with *EcoRI* to reveal the ligated product and with *PstI* to ascertain orientation with respect to the promoter. The products of the digestion were resolved on a 1.6% agarose gel by electrophoresis at 200 volts for 2 hours.

The plasmid that contained the 2-cistron fragment and had the correct orientation (see 3.1.5) was designated pQR163 (see Fig. 3.1A).

The recombinant *E. coli* host that contained pQR163 was further grown on agar plates containing ampicillin overnight at 37 °C. Colonies were removed from the plate into sterile glycerol (20% v/v) for storage at -70 °C.

### **2.19 Induction of *E. coli* cultures bearing pQR163 with IPTG.**

Nutrient broth (1-5 litres) was prepared as in section 2.2. The sterile medium was placed at room temperature for 1-2 hours to cool down before adding ampicillin (0.1 mg/ml). An aliquot (20  $\mu$ l) of pQR163 or pKK223.3 (used as control) in 20% (v/v) glycerol was inoculated into 1.25 litres of medium (contained in a 5 litre flask). The cells were grown for 4-5 hours at 28 °C. An aliquot (1ml) was removed and growth was assessed by measuring the optical density at 550 nm. Inducer (IPTG) was added

to a final concentration of 0.5 mM when growth was in the exponential phase ( $OD_{550} = 2-3$ ). Growth of cells after addition of IPTG was continued overnight at 28 °C.

## 2.20 Isolation of periplasmic proteins.

The release of the periplasmic contents was carried out using a modified spheroplasting/osmotic shock procedure developed by C. French *et al.*, (unpublished).

*E. coli* cells from an overnight culture (5 litres) were pelleted by centrifugation at 8300 x g (ave) for 10 min. at 4 °C. The cell pellet was resuspended in <sup>60 ml of</sup> 200 mM Tris/HCl pH 7.5, 20% (w/v) sucrose (ribonuclease free), 1 mM Na<sub>2</sub>EDTA and lysozyme (500 µg/ml). The suspension was left for 15 min. at room temperature. An osmotic shock was produced by adding an equal volume of sterile water and mixing thoroughly. The mixture was left for 15 min. at room temperature. Spheroplasts were pelleted by centrifugation at 100000 x g (ave) for 90 min. at 10 °C. The supernatant containing the periplasmic proteins was transferred to a sterile Falcon tube.

## 2.21 Determination of protein concentration. Bradford assay.

Protein concentration was determined according to the method of Bradford (1976). A standard curve was prepared in a total volume of 800 µl containing 1-20 µg of commercial ribonuclease A. For measurements of protein samples, aliquots (2 µl) were diluted to 800 µl with sterile water. Protein concentration was determined by adding 200 µl of Bio-rad protein assay reagent and reaction was carried out for 30 min. at room temperature. Assays were carried out in triplicate. The optical density at 595 nm of the standards and of the sample was measured. The values obtained from the standards were used to plot a standard curve ( $OD_{595}$  versus concentration of ribonuclease). The concentration of the sample was determined by extrapolation using the standard curve.

## **2.22 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE).**

Polyacrylamide gels were used to separate proteins under denaturing conditions according to their molecular weight.

### **2.22.1 Preparation of the resolving gel (16% v/v).**

A stock solution of acrylamide (30% w/v) was prepared by dissolving acrylamide (30 g) and bisacrylamide (0.8 g) in 100 ml of distilled water (Laemmli, 1970). The resolving gel was prepared by mixing stock acrylamide (16 ml) with 1.5 mM Tris/HCl pH 8.8 (7.5 ml), 10% (w/v) SDS (0.3 ml), 10% (w/v) ammonium persulfate (0.1 ml) and sterile water (6.1 ml).

### **2.22.2 Preparation of the stacking gel (3% v/v).**

The stacking gel was prepared by mixing stock acrylamide (1 ml) with 0.5 M Tris/HCl pH 6.8 (2.5 ml), 10% (w/v) SDS (0.1 ml), 10% (w/v) ammonium persulfate (0.1 ml) and sterile water (6.3 ml)

### **2.22.3 Electrophoresis.**

For treatment of the glass plates refer to 2.16.1

A home made electrophoresis tank with top and bottom reservoirs was used for electrophoresis. Glass plates of the appropriate size were assembled separated by 1.5 mm spacers and clamped together with bulldog clips.

The polymerization of the resolving gel solution was catalyzed by the addition of 8.75  $\mu$ l of TEMED. The solution was immediately poured to a height of 2 cm from the top of the glass plates. A small volume of isopropanol was applied to the top of the solution to ensure a level surface of polymerization. Once the gel had polymerized, the isopropanol was poured off and a 10-spacer comb was introduced between the glass plates.

The polymerization of the stacking gel solution was catalyzed by the addition of 7.5  $\mu$ l of TEMED. The solution was then immediately poured on top of the polymerized resolving gel using a Pasteur pipette. Once polymerized, the comb was removed and the wells washed with running buffer (25 mM Tris base pH 8.3, 190 mM glycine, 0.1% (w/v) SDS). The gel was then placed in the electrophoresis tank and the top and bottom reservoirs were filled with running buffer.

#### **2.22.4 Treatment of protein samples prior to electrophoresis.**

The concentration of protein samples (0.2-20  $\mu$ g) was adjusted prior to electrophoresis by adding (as minimum) an equal volume of sample buffer (62.5 mM Tris/HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.00125% (w/v) bromophenol blue). The samples were then boiled for 2-3 min. at 92 °C, and 2-mercaptoethanol (2  $\mu$ l) was added. Protein samples were loaded onto the polyacrylamide gel in volumes of 50-100  $\mu$ l. Electrophoresis was carried out at 30 mA constant current. The current was increased to 40 mA once the dye front had entered the resolving gel. Electrophoresis was stopped when the dye front reached the bottom of the resolving gel.

#### **2.22.5 Silver staining.**

Silver staining of gels was carried out using a commercial silver staining kit purchased from Sigma. All steps were carried out at room temperature. Staining was initiated by fixing the gel in a solution of methanol : acetic acid (3:1) (300 ml, 20 min., x 3). The gel was then rinsed (3x) in 300 ml of distilled water for 10 min. with rocking. Staining was carried out using silver nitrate solution for 30 min. with constant agitation. The gel was briefly rinsed with distilled water (300 ml) for 10 seconds prior to developing the stain.

Protein bands were visualized by adding 150 ml of pre-warmed (25 °C) developer solution. The gel was agitated in developer for 10-15 min. until the bands just begin to appear. The developer solution was then poured off and fresh developer (150 ml)

was added to fully develop the protein bands for 5-8 min. with agitation. The developing process was stopped by agitating the gel in a solution of 1% (v/v) acetic acid (300 ml) for 5 min. The background was eliminated by agitating the gel in a reducer solution (300 ml) for 10-30 seconds. The gel was then rinsed under tap water for 1 min. followed by three rinses in distilled water (300 ml) for 10 min. Gels were stored sealed in Saran wrap.

### **2.23 Purification of recombinant bovine pancreatic ribonuclease A (RNase). Fast protein liquid chromatography (FPLC).**

RNase has a net positive charge at pH 6.5. Thus, its purification is extremely easy using cation exchange chromatography.

#### **2.23.1 Sulfonic acid (S)-Sepharose Fast Flow (FF, cation exchanger).**

This type of chromatography was used to separate all positively charged proteins from the pool of periplasmic proteins (see 2.20). The column used was purchased from Pharmacia-LKB (column XK 16/40, 16 mm x 40 cm). This column is compatible with the FPLC. The gel used was S-Sepharose FF (Pharmacia-LKB).

The gel bed dimensions were 16 mm x 25 cm and the column was equilibrated with 50 mM 2-[N-morpholino] ethane sulfonic acid (MES) pH 6.5 (250 ml), 50 mM MES pH 6.5, 1 M NaCl (500 ml) and finally with 50 mM MES pH 6.5 (250 ml). The column was filled with 20% (v/v) ethanol for storage at 4 °C.

The periplasmic extract (see 2.20) was filtered through a cellulose nitrate filter (pore size, 0.45  $\mu$ m) and loaded onto the column using a peristaltic pump (P1, Pharmacia-LKB) at a flow rate of 4 ml/min. The column was then washed with 50 mM MES pH 6.5 (250 ml) followed by isocratic elution with 50 mM MES pH 6.5, 1 M NaCl (250 ml) to elute bound proteins at a flow rate of 4 ml/min. Finally the column was washed with 50 mM MES pH 6.5 (250 ml). Fractions (12.5 ml) were collected. The

peak obtained after isocratic elution, which contains positively charged proteins, was collected and concentrated using an ultrafiltration cell (see 2.32.2).

### **2.23.2 Ultrafiltration using YM-10 membranes.**

The fractions that contained positively charged proteins (see 2.23.1) were pooled in a ultrafiltration cell (Amicon, model 8050). Filtration of the sample was carried out using a YM-10 ultrafiltration membrane (Amicon) under helium pressure (2 bar) with stirring. The volume of the retentate containing positively charged proteins of molecular weight greater than 10000 was measured and the volume adjusted to 2.5 ml with sterile water. The retentate was desalted into sterile water using a NAP-25 column (DNA grade, Sephadex G-25, Pharmacia-LKB). The final volume of the eluate was 3.5 ml.

### **2.23.3 Mono-S HR 5/5 column (cation exchanger).**

Recombinant RNase was purified from the desalted pool of positively charged proteins by FPLC, using a strong cation exchanger resin (Mono-S HR 5/5, Pharmacia-LKB) column.

Elution was carried out using buffer A, 50 mM MES pH 6.5 and buffer B, 50 mM MES pH 6.5, 1 M NaCl. A gradient was applied of 17.5 mM [B]/min. at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected. The peak that showed ribonuclease activity was collected and desalted on a G-25 column (NAP-5, Pharmacia-LKB). Recombinant RNase was eluted in 1 ml of sterile water and concentrated using a microconcentrator (Centricon-10, Amicon). The retentate (70-100  $\mu$ l) was transferred to a sterile tube and stored at -20 °C.

### **2.24 N-terminus sequencing of recombinant RNase.**

The first 20 amino acids of recombinant RNase expressed in *E. coli* were sequenced using an Applied Biosystems 470A automated gas phase peptide sequencer. The

method used was Edman degradation with identification of the phenylthiohydantoin amino acid derivatives by high pressure liquid chromatography (HPLC).

## **2.25 Site-directed mutagenesis (SDM) of recombinant RNase using recombinant circle polymerase chain reaction (RCPCR).**

RCPCR (Jones and Howard, 1990) is based on two separate PCR assays. Synthesis, elution and deprotection purification and determination of primer concentration were carried out as described in 2.13.2, 2.13.3, 2.13.4.1 and 2.13.5 respectively. All PCR assays had 50 pmols of each primer involved.

### **2.25.1 Design of primers.**

The primers for RCPCR were designed such that when they are used in PCR reactions, the products generated are double stranded molecules with cohesive ends that upon combination, denaturation and reannealing produce “nicked” or “gapped” circles ready to transfect (see Fig. 2.1). The “break point” between the two 5’ ends of the primers in one PCR reaction (**a** and **b** in Fig. 2.1) are different from the “break point” in the other PCR reaction (**c** and **d** in Fig. 2.1).

### **2.25.2 Mutations.**

#### **2.25.2.1 Mutation of Lys-41 to Arg.**

Four oligonucleotides were synthesized and designated as primers 60, 70, 80 and 90. Primer 60 contained the codon for Arg at position 41 of RNase and primer 70 contained its complementary sequence.

Primer 60: 5' -AAGATCGATGCC**CGT**CCAGTGAACACCTTTG -3'

Primer 70: 5' -GTGTTCACTGG**ACG**GATCGATCTTTGGTC -3'

Primer 80: 5' -TGGTCAGGTTCCGGCTCTTCATCATCTGGT -3'

Primer 90: 5' -CTTTGTGCACGAGTCCCTGGCTGATGTCCA- 3'

**CGT**: Codon for Arg at position 41 of RNase.

**ACG**: Complementary sequence.

Two PCR incubations were set up. One incubation contained primers 60 and 80 (a and b in Fig. 2.1) and the other contained primers 70 and 90 (c and d in Fig. 2.1). In both PCR incubations pQR162 (see Fig. 3.1A) was used as template (500 ng), nucleotides (0.2 mM), 20 mM Tris/HCl pH 8.8, 15 mM  $(\text{NH}_4)_2\text{SO}_4$ , 2 mM  $\text{MgCl}_2$ , 0.05% (v/v) NP40 (detergent), 0.05% (v/v) Tween 20 (detergent) in a total volume of 100  $\mu\text{l}$ . The reaction components were centrifuged at 10000 x g (ave) for 2 seconds at room temperature. An equal volume of paraffin oil was added to avoid evaporation during the reaction. Template DNA was fully denatured by incubation for 5 min. at 92 °C and the reaction was initiated by the addition of Tet/Z polymerase (2 units, 1 unit/ $\mu\text{l}$ ). The reaction was allowed to undergo 25 cycles in a thermal cycler (Techne 2000). See section 4.2.2 for conditions of the PCR incubations.

Both PCR incubations (100  $\mu\text{l}$ ) were transferred to sterile eppendorf tubes. Stop mix (25  $\mu\text{l}$ , see 2.10) was then added and the samples loaded onto a 1% agarose gel (see 2.9). Electrophoresis was carried out for 2 hours at 180 volts. The DNA bands were then visualized under a UV transilluminator. The DNA fragment produced from each PCR reaction was extracted from the gel (see 2.17) and ethanol precipitated (see 2.7). Each pellet was resuspended in 20  $\mu\text{l}$ . An aliquot (10  $\mu\text{l}$ ) of each purified DNA was transferred to a sterile eppendorf tube and mixed with 5  $\mu\text{l}$  of 10 x annealing buffer (100 mM Tris/HCl pH 8.8, 1 M NaCl and 10 mM  $\text{Na}_2\text{EDTA}$ ). The volume was adjusted to 50  $\mu\text{l}$  with sterile water. The combined DNA fragments were denatured for 5 min. at 92 °C and reannealed for 2 hours at 55 °C (see Fig.2.1).

The “nicked circles” (see Fig. 2.1) were used to transform an aliquot of competent cells (see 2.4 and 2.5). The transformed cells were inoculated into 5 ml of nutrient broth and grown for 3 hours at 37 °C with shaking. An aliquot (100  $\mu\text{l}$ ) was spread onto agar plates containing ampicillin and grown overnight at 37 °C. Single colonies were further grown in agar plates containing ampicillin and in 5 ml of nutrient broth. Mini-preps were carried out (see 2.8.1) to isolate the plasmid. This plasmid was used as a template for double stranded DNA sequencing (see 2.16).



The plasmid containing the mutation was designated pQR164. The 2-cistron fragment of pQR164 was subcloned into pKK223.3 (see 2.18) and the resulting plasmid chimaera was named pQR165.

### 2.25.2.2 Mutation of Gln-69 to Ala

Four oligonucleotides were synthesized and designated as primers 100, 101, 102 and 103. Primer 100 contained the codon for Ala at position 69 of RNase and primer 102 contained its complementary sequence.

Primer 100: 5' -TGCAAGAATGGGG**GCT**ACCAATTGCTACCAG 3'

Primer 101: 5' -GGCAACATTTTTCTGGGAGCACACGGCCTG -3'

Primer 102: 5' -GTAGCAATTGGT**AGC**CCCATTCTTGCAGGC -3'

Primer 103: 5' -TCCACCATGAGCATCACCGACTGCCGTGAG -3'

**GCT**: Codon for Ala at position 69 of RNase.

**AGC**: Complementary sequence.

Two PCR incubations were set up. One incubation contained primers 100 and 101 (a and b in Fig. 2.1) and the other contained primers 102 and 103 (c and d in Fig. 2.1). In both PCR incubations pQR162 was used as template (20 ng), nucleotides (0.2 mM), 20 mM Tris/HCl pH 8.8, 10 mM KCl, 6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 0.1% (v/v) Triton X-100 and 0.1 mg/ml (w/v) of nuclease-free BSA in a total volume of 100  $\mu$ l. The reaction components were centrifuged at 10000 x g (ave) for 2 seconds at room temperature. An equal volume of paraffin oil was added to avoid evaporation during the reaction. Template DNA was fully denatured by incubation for 5 min. at 92 °C and the reaction was initiated by the addition of *pfu* polymerase (1  $\mu$ l, 2.5 units/ $\mu$ l). The reaction was allowed to undergo 25 cycles in a thermal cycler (Techne 2000). See section 4.3.2 for conditions of the PCR incubations.

The rest of the procedure is as described in section 2.25.2.1 but the annealing temperature used for the combined gel purified DNA fragments was 57 °C (see Fig. 2.1). The plasmid containing the mutation was designated pQR166. The 2-cistron fragment of pQR166 was subcloned into pKK223.3 (see 2.18) and the resulting plasmid chimaera was named pQR167.

### **2.25.2.3 Mutation of Asn-71 to Ala.**

Two oligonucleotides were synthesized and designated as primers 104 and 105. Primer 104 contained the codon for Ala at position 71 of RNase and primer 105 its complementary sequence.

Primer 104: 5' -AATGGGCAGACCGCTTGCTACCAGAGCTAC -3'

Primer 105: 5' -GCTCTGGTAGCAAGCGGTCTGCCCATTTCTT -3'

**GCT:** Codon for Ala at position 71 of RNase.

**AGC:** Complementary sequence.

Two PCR incubations were set up. One incubation contained primers 104 and 101 (see 2.25.2.2) (a and b in Fig. 2.1) and the other contained primers 105 and 103 (see 2.25.2.2) (c and d in Fig. 2.1). In both PCR incubations pQR162 was used as template (2 ng). The components and procedures utilized are as described in section 2.25.2.2. See section 4.4.2 for PCR conditions. The annealing temperature used for the combined gel purified DNA fragments was 55 °C (see Fig. 2.1). The plasmid containing the mutation was designated pQR170. The 2-cistron fragment of pQR170 was subcloned into pKK223.3 (see 2.18) and the resulting plasmid chimaera was named pQR171.

#### **2.25.2.4 Mutation of Gln-69 and Asn-71 to Ala and Ala.**

Two oligonucleotides were synthesized and designated as primers 106 and 107. Primer 106 contained the codons for Ala at position 69 and 71 of RNase and primer 107 their complementary sequences.

Primer 106: 5' -TGCAAGAATGGGG**GCTACCGCT**TGCTACCAGAGCTAC -3'

Primer 107: 5' -GCTCTGGTAGCA**AGCGGTAGCCCCATTCTTGCAGGC** -3'

**GCT**: Codons for Ala at position 69 and 71 of RNase.

**AGC**: Complementary sequences.

Two PCR incubations were set up. One incubation contained primers 106 and 101 (see 2.25.2.2) (a and b in Fig. 2.1) and the other contained primers 107 and 103 (see 2.25.2.2) (c and d in Fig. 2.1). In both PCR incubations pQR162 was used as template (2 ng). The components and procedures utilized are as described in section 2.25.2.2. See section 4.5.2 for PCR conditions. The annealing temperature used for the combined gel purified DNA fragments was 55 °C (see Fig. 2.1). The plasmid containing the mutation was designated pQR168. The 2-cistron fragment of pQR168 was subcloned into pKK223.3 (see 2.18) and the resulting plasmid chimaera was named pQR169.

#### **2.25.2.5 Mutation of Glu-111 to Gln.**

Four oligonucleotides were synthesized and designated as primers 120, 121, 122 and 123. Primer 120 contained the codon for Gln at position 111 of RNase and primer 122 contained its complementary sequence.

Primer 120: 5' -ATTGTGGCTTGT**CAG**GGAAACCCGTACGTG -3'

Primer 121: 5' -GTGTTTATTCGCCTGGGTGGTCTTGTAGGC -3'

Primer 122: 5' -GTACGGGTTTCC**CTG**ACAAGCCACAATGAT -3'

Primer 123: 5' -CCAGTCCACTTTGATGCTTCAGTGTAGGTC -3'

**CAG:** Codon for Gln at position 111 of RNase.

**CTG:** Complementary sequence.

Two PCR incubations were set up. One incubation contained primers 120 and 121 (see 2.25.2.2) (a and b in Fig. 2.1) and the other contained primers 122 and 123 (see 2.25.2.2) (c and d in Fig. 2.1). In both PCR incubations pQR162 was used as template (2 ng). The components and procedures utilized are as described in section 2.25.2.2. See section 4.6.2 for PCR conditions. The annealing temperature used for the combined gel purified DNA fragments was 55 °C (see Fig. 2.1). The plasmid containing the mutation was designated pQR172. The 2-cistron fragment of pQR168 was subcloned into pKK223.3 (see 2.18) and the resulting plasmid chimaera was named pQR173.

## **2.26 Ribonuclease assays.**

### **2.26.1 Qualitative RNA plate assay.**

A solution containing 2% (w/v) agar powder (Bacto-agar), 100 mM MES pH 6.5 and 0.3% (w/v) yeast RNA (100 ml) was autoclaved, poured into 4 Petri dishes, and allowed to solidify. Round holes were made in the RNA agar using a sterile cork-borer and recombinant or commercial RNase or different RNase mutants (0.1-25 µg) were added in a volume of 100 µl. The plates were incubated for 2-4 hours at 37 °C. Ribonuclease activity was visualized by soaking the plate with 2 M HCl. The addition of acid precipitates non-hydrolyzed RNA, thus clear zones around a well indicate RNase activity.

### **2.26.2 Total hydrolysis of cytidine-2':3'-cyclic phosphate.**

The hydrolysis of cytidine-2':3'-cyclic phosphate by RNase was measured using the method of Crook *et al.*, (1960). The assay was carried out in a volume of 1 ml in a cuvette of 1 cm path length (Hellma) and contained 0.3 mM cytidine-2':3'-cyclic phosphate in 0.1 M Tris acetate pH 6.5 ( $I=0.1$ ). The reaction was initiated by addition of RNase to a final concentration of 2.9 µM.

A spectra of cytidine-2':3'-cyclic phosphate was obtained over a wavelength range of 190-300 nm, against a blank containing buffer alone. Enzyme was then added to the sample cuvette and hydrolysis was allowed to continue at room temperature for 2 hours. The reaction was assumed to be complete at this time and a spectrum of the product cytidine 3'-phosphate was obtained.

### **2.26.3 Difference spectra of the hydrolysis of dinucleotide substrates.**

The hydrolysis of CpA, CpG, CpC or CpU by RNase was measured as follows: all assays were carried out in a volume of 1 ml in a cuvette of 1 cm path length (Hellma) and contained 87  $\mu\text{M}$  CpA, 42.5  $\mu\text{M}$  CpG, 45  $\mu\text{M}$  CpC or 45  $\mu\text{M}$  CpU respectively in 0.1 M Tris acetate pH 6.5 ( $I=0.1$ ). Reactions were initiated by the addition of RNase to a final concentration of  $1.4 \times 10^{-7}$  M

Spectra of CpA, CpG, CpC and CpU were obtained over a wavelength range of 190-300 nm, against a blank containing buffer alone. Enzyme was then added to the sample cuvette and hydrolysis was allowed to continue for 10 minutes. At this time, a spectrum was obtained of the reaction products.

### **2.26.4 Spectrophotometric assay of cytidyl (3'-5') - adenosine (CpA) hydrolysis.**

The hydrolysis of CpA by RNase was measured at room temperature in cuvettes of 0.1 cm path length (Hellma) in a total volume of 250  $\mu\text{l}$ . Reactions contained varying concentrations of CpA in 0.1 M (1,3-bis[tris(hydroxymethyl)-methylamino] propane) pH 7.0, 50 mM NaCl ( $I=0.1$ ) and were initiated by the addition of RNase to a final concentration of  $8.77 \times 10^{-10}$  M when using commercial or recombinant RNase. The final concentration of the mutant enzymes used in these assays varies as described in Chapter 5.

The hydrolysis of CpA was measured, against a blank containing 0.1 M (1,3-bis[tris(hydroxymethyl)-methylamino] propane) pH 7.0, 50 mM NaCl ( $l=0.1$ ) and CpA by a decrease in absorbance with time at 286 nm.

#### **2.26.5 Spectrophotometric assay of cytidine-2':3'- cyclic phosphate hydrolysis.**

The conditions of the assay were as described above (see 2.26.4), but containing differing amounts of cytidine-2':3'- cyclic phosphate. The reaction was initiated by the addition of RNase to a final concentration of  $2.9 \times 10^{-7}$  M.

The hydrolysis of cytidine-2':3'-cyclic phosphate was measured against a blank containing buffer and substrate by an increase in absorbance with time at 286 nm.

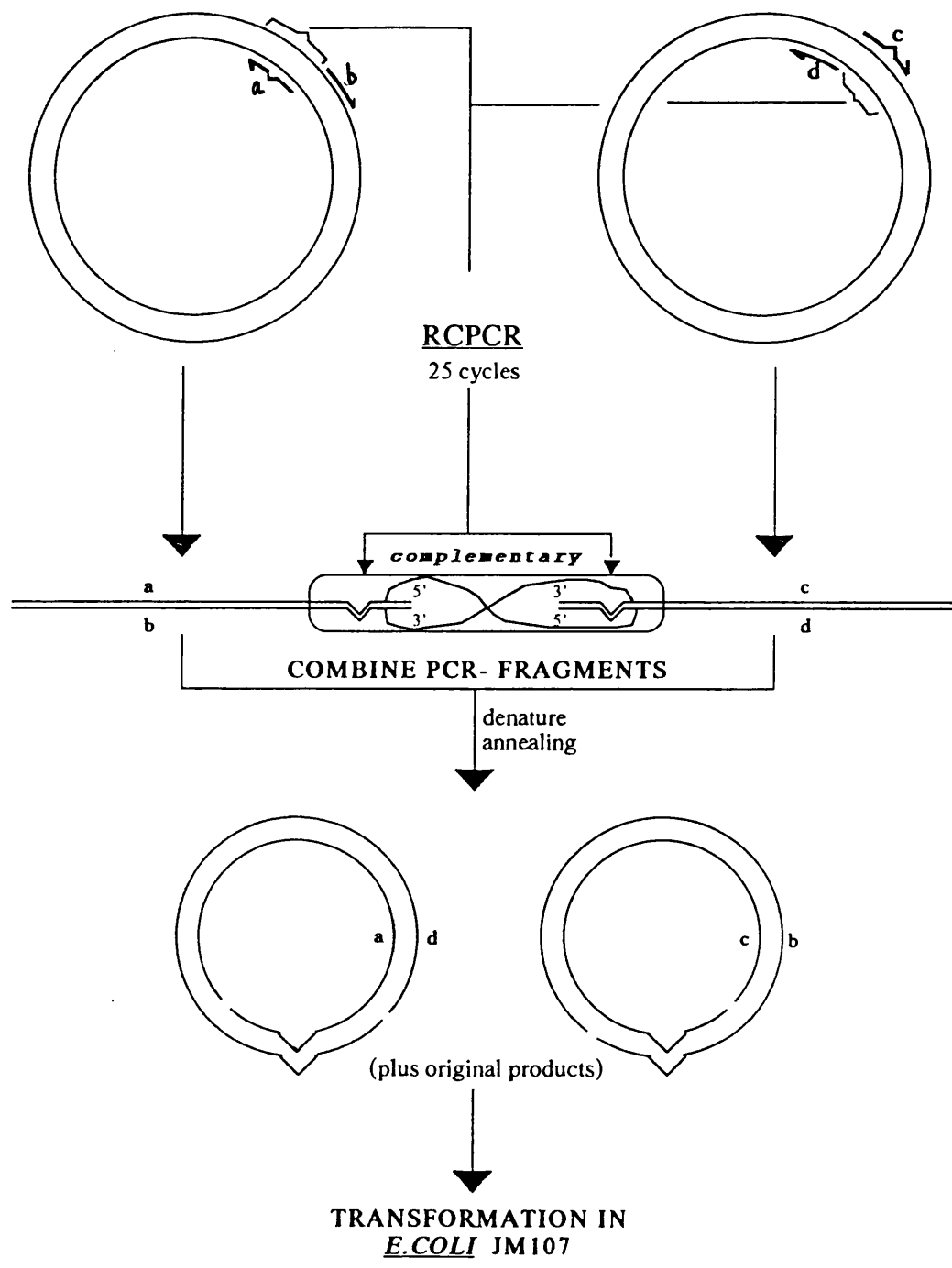
#### **2.26.6 Spectrophotometric assay of CpG, CpC and CpU hydrolysis.**

The hydrolysis of CpG, CpC or CpU by RNase was measured in cuvettes of 0.1 cm path length (Hellma) in a total volume of 250  $\mu$ l. Reaction contained 0.2 mM CpG, CpC or CpU in 0.1 M Tris acetate pH 6.5 ( $l= 0.1$ ). The reaction was initiated by the addition of commercial, recombinant or mutant RNases to varying concentrations (see Chapter 5).

The hydrolysis of the dinucleotides was measured against a blank containing buffer and substrate by an increase in absorbance with time at 265 nm.

#### **2.27 Densitometry.**

A polyacrylamide gel was photographed and the negative was used to scan the density of the band of interest by light reflection using a Cromoscan 3 densitometer (Joyce Lobel Ltd.).



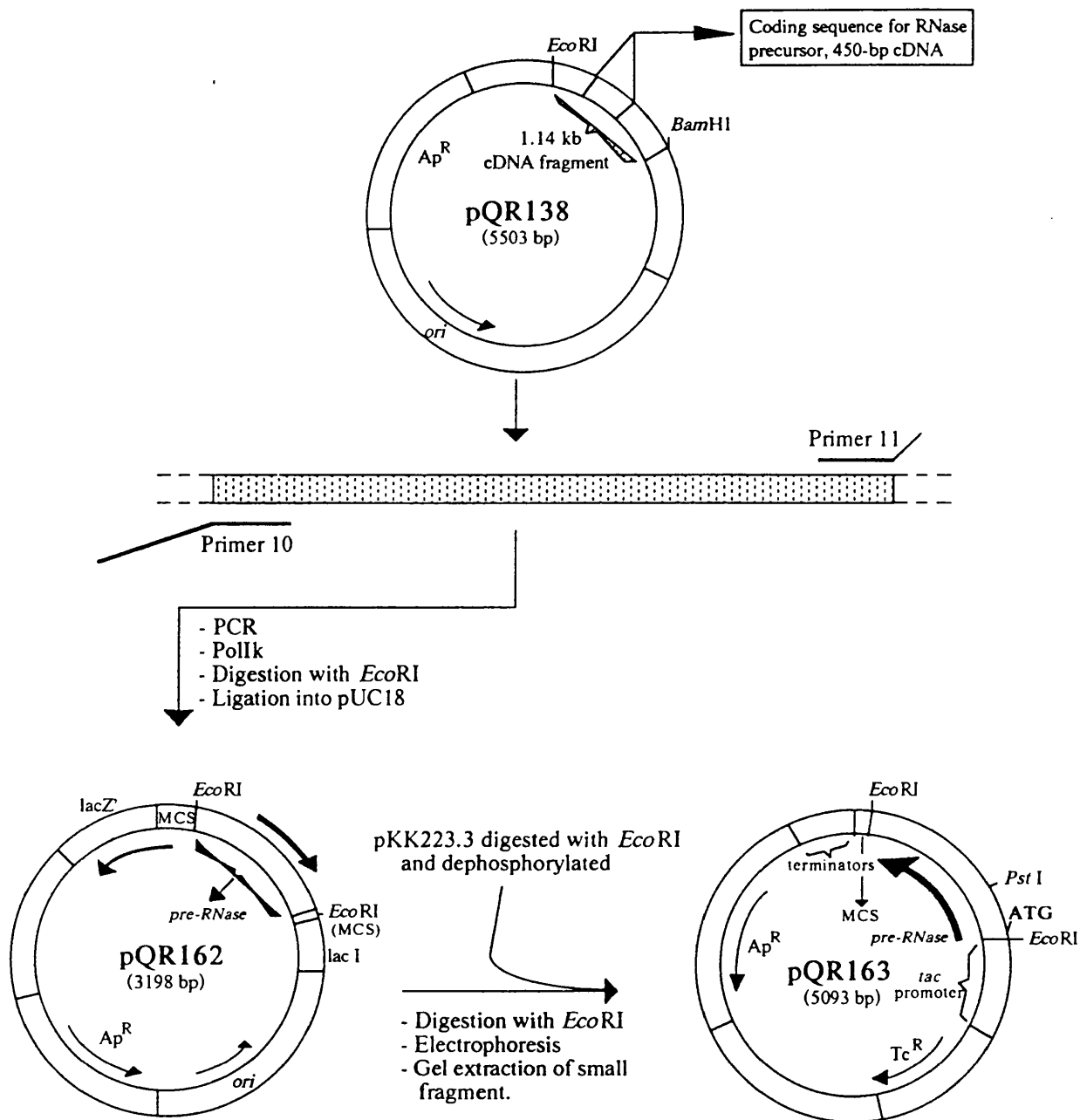
**Figure 2.1:** Site-directed mutagenesis using RCPCR

### **CHAPTER 3: CLONING, EXPRESSION AND PURIFICATION OF BOVINE PANCREATIC RIBONUCLEASE (RNase) IN *ESCHERICHIA COLI*.**

The coding sequence for bovine pancreatic ribonuclease A (RNase) precursor has been cloned and expressed in *E.coli* using the polymerase chain reaction (PCR) technique. A PCR incubation was performed utilizing a template comprising the plasmid chimaera pQR138, which contains the coding sequence for the RNase precursor, and primers that allow for the addition of new sequences at the 5' and 3' ends of the coding sequence. The resulting fragment contains two coding sequences, one for a hexapeptide and the other for pre-ribonuclease. This fragment has been cloned into the expression vector pKK223.3 under the control of the *tac* promoter, to form a 2-cistron vector. Upon induction with IPTG, *E. coli* cells harboring this construct generate a bicistronic mRNA which upon translation produces a hexapeptide and pre-ribonuclease. The ribonuclease precursor is efficiently translocated into the periplasmic space of *E.coli*. In the process the signal sequence is removed thus generating mature ribonuclease. Formation of the disulfide bridges in ribonuclease is facilitated by the oxidative environment of the periplasm and fully active protein is obtained.

Produced recombinant RNase has been purified to homogeneity by cation exchange chromatography and the removal of the signal sequence has been verified by N-terminus sequencing. The total process from inoculation of medium to obtaining pure and fully active recombinant RNase is achieved in 48 hours.





**Figure 3.1A: New constructs.**

Plasmid pQR138 serves as a template for a PCR incubation using primers 10 and 11 (see Fig. 3.1B). The PCR fragment thus produced is ligated into digested and dephosphorylated pUC18 forming a plasmid chimaera designated pQR162. This plasmid is digested with *EcoRI*. The products of the digestion are electrophoresed and the two-cistron fragment is gel purified and ligated into digested and dephosphorylated pKK223.3 to form pQR163.

### 3.1 Production of an expressible coding sequence for RNase using the polymerase chain reaction (PCR) technique.

The plasmid chimaera pQR138 (see Fig. 3.1A) contains a 1.14 Kb *EcoRI/BamHI* cDNA fragment. This fragment contains the complete coding sequence for RNase precursor (450 bp) (Carsana *et al.*, 1988). However, the ligation of the 1.14 Kb fragment into an expression vector would produce an inefficient construct for the expression of RNase. The ribosome binding site (RBS) provided by the vector and the initiation codon of RNase precursor are too far apart (Carsana *et al.*, 1988) for translation of the messenger RNA to occur. To overcome this problem, primers can be synthesized complementary to the 5' and 3' ends of the coding sequence for RNase precursor and used in a PCR incubation with pQR138 as template. The resulting PCR fragment could then be ligated into an expression vector creating a construct in which the distance between the RBS and the initiation codon is optimal for translation of the messenger RNA (Winnacker, 1987).

Nevertheless, there are also other factors that can hinder the expression of eukaryotic proteins in prokaryotes. These include degradation of the protein (Maurizi *et al.*, 1985), formation of inclusion bodies due to insolubility of the product (McGeehan *et al.*, 1989), and instability of the messenger RNA (Newbury *et al.*, 1987). Another problem encountered is that of secondary structure of the messenger RNA (Hall *et al.*, 1982). Preliminary studies using Beckman Microgenie software to simulate the secondary structure of the mRNA that would be produced following ligation of the ribonuclease precursor coding sequence into an expression vector such as pKK223.3, indicated that efficient expression would not be possible due to the formation of a stem loop containing the RBS and the initiation codon for pre-ribonuclease.

Similar problems were encountered by Schoner *et al.*, (1984) when trying to express methionyl bovine growth hormone (Met-bGH). A series of DNA sequences coding for discrete polypeptides were fused to the coding sequence of Met-bGH. The different constructs, or 2-cistron vectors (the mRNA produced is bicistronic) were expressed

and the yields of Met-bGH increased in certain cases. Mashko *et al.*, (1990) has also made 2-cistron constructs in order to solve translational problems. In consequence, a strategy using PCR, has been developed to incorporate a coding sequence for a hexapeptide at the 5' end of the coding sequence for pre-RNase in order to produce an expressible sequence which has been inserted into pKK223.3 forming a 2-cistron vector. Microgenie software has been used to show that this construct minimizes the secondary structure of the messenger RNA.

### **3.1.1 Design of the primers for PCR.**

To obtain an expressible coding sequence for the RNase precursor from pQR138 (see Fig. 3.1A), primers were synthesized complementary to the 5' and 3' ends of the coding sequence for RNase precursor (see Fig. 3.1B) and used in a PCR incubation. The characteristics of the primers, designated 10 and 11, are explained in 2.13.1. The resultant DNA fragment from a PCR incubation utilizing pQR138 as template and primer 10 and 11, comprises 2 sets of coding sequences. One set contains the coding sequence for a hexapeptide (first cistron) and the other for RNase precursor (second cistron).

Transcription of this fragment produces bicistronic mRNA which upon translation generates a hexapeptide and the RNase precursor. Therefore, the DNA fragment obtained after a PCR incubation using primers 10 and 11 has been designated "2-cistron fragment".

### **3.1.2 Generation of a 2-cistron fragment using PCR.**

A PCR incubation using pQR138 as template and primers 10 and 11 was carried out (see 2.13.6). The incubation was allowed to undergo 25 cycles of denaturation at 92° C for 1.5 min., annealing at 55 °C for 1 min., and extension at 72 °C for 1.5 min., in a thermal cycler (Techne 2000). To analyze the PCR products, an aliquot (10%) of the reaction was loaded onto an agarose gel (see 2.9) and electrophoresed. A single DNA band, the 2-cistron fragment, was observed upon UV irradiation of the gel.



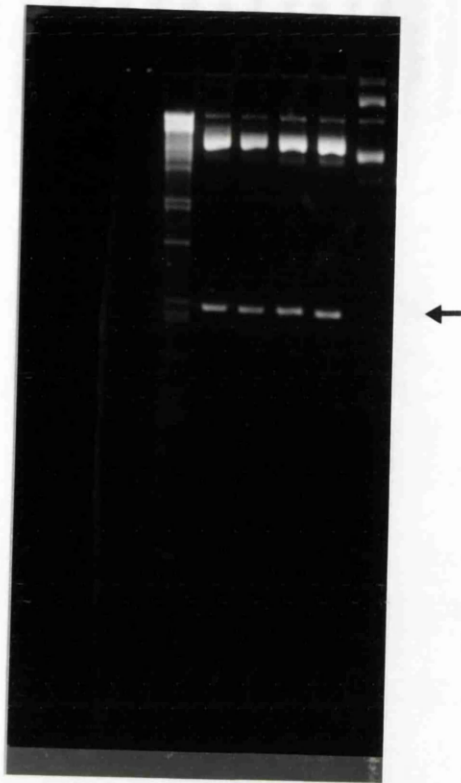
### **3.1.3 Ligation of the 2-cistron fragment into pUC18.**

The characteristics of pUC18 are explained in 2.15. The 2-cistron DNA fragment was further treated with *Pollk* as described in 2.14. to repair ragged ends, and then cleaved with *EcoRI* (20 units) for 3 hours at 37 °C (see 2.10) prior to ligation into previously digested and dephosphorylated pUC18 (see 2.11). Ligation was left to proceed overnight at room temperature (see 2.12). Ligated products were added to competent cells and transformation was carried out (see 2.4 and 2.5). An aliquot of the transformation mixture (100 µl) was spread onto ampicillin, IPTG and X-Gal agar plates (see 2.2). The recombinants were grown overnight at 37 °C. Due to the fact that the vector was dephosphorylated, thereby preventing re-ligation, a higher proportion of white colonies over blue colonies was obtained.

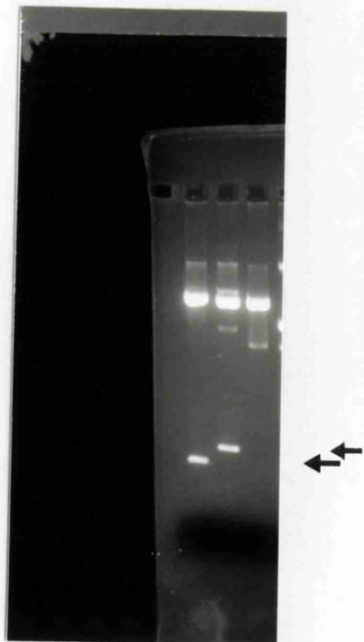
Four white colonies were selected and further grown on similarly supplemented agar plates and also in 5 ml of nutrient broth. Mini-preps were carried out from each recombinant to isolate plasmid DNA (see 2.8.1). The insert that was contained in each plasmid was revealed upon digestion with *EcoRI*. The products of the different digestions were resolved by electrophoresis on a 1.6% agarose gel containing ethidium bromide (see Fig. 3.2). All four recombinant colonies selected contained the plasmid with the 2-cistron fragment inserted in the *EcoRI* site. One of these recombinants was further grown in 400 ml of nutrient broth overnight at 37 °C with shaking. Large scale isolation of plasmid DNA was carried by CsCl gradient centrifugation (see 2.8.2). This plasmid was used as a template for double stranded DNA sequencing

### **3.1.4 Verification of the sequence by double stranded DNA sequencing.**

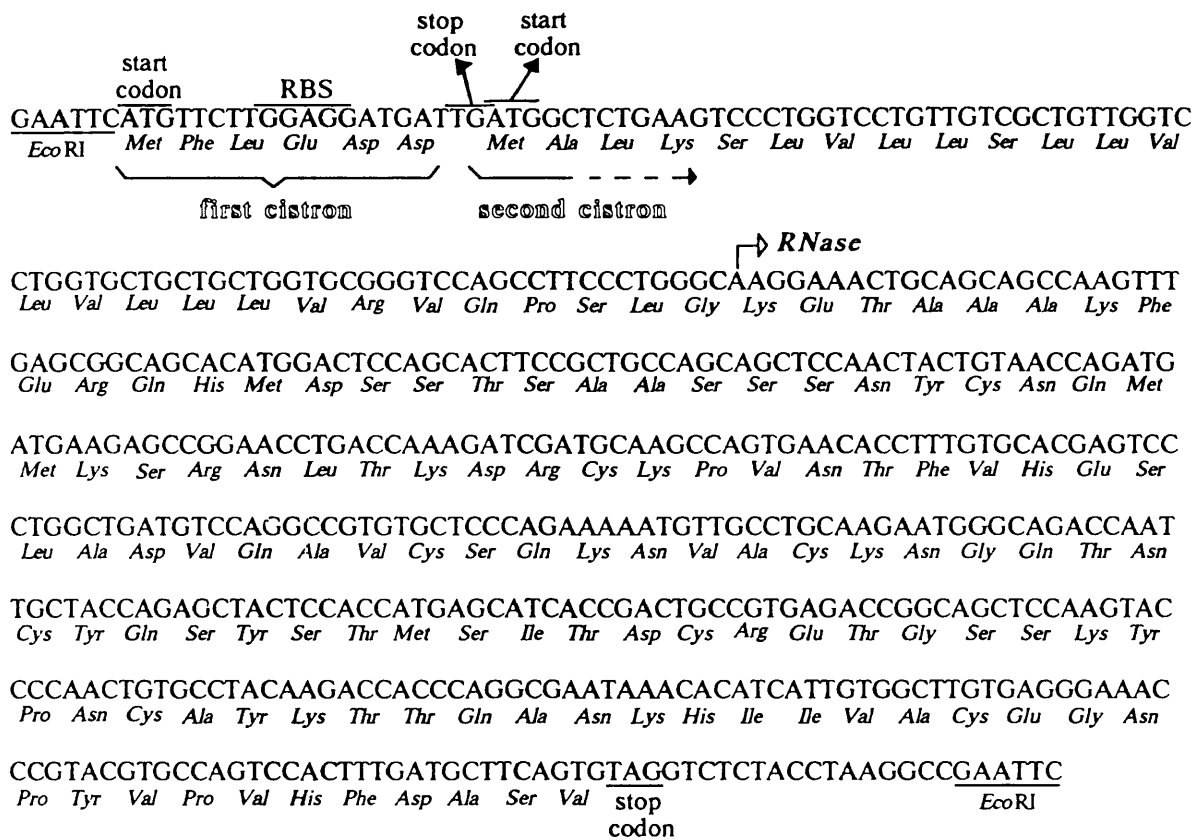
To obtain the sequence of the 2-cistron fragment ligated into pUC18, double stranded DNA sequencing was carried out (see 2.16.5). The nucleotide and amino acid sequence are shown in Fig. 3.4. The plasmid containing the 2-cistron fragment was designated pQR162 (see Fig. 3.1A).



**Figure 3.2:** Products of the digestion with *EcoRI* to reveal the two-cistron fragment.



**Figure 3.3:** Products of the digestion of pQR163 with *EcoRI* to reveal the insert (top arrow) and products of the digestion of pQR163 with *PstI* (bottom arrow). The production of a DNA fragment after digestion with *PstI*, indicates correct orientation of the RNase gene with respect to the *tac* promoter.



**Figure 3.4:** Sequence of the two-cistron DNA fragment obtained by double stranded DNA sequencing using pQR162 as template (see Fig 3.1A). The aminoacid sequence shown corresponds to a hexapeptide (first cistron) and to pre-ribonuclease (second cistron).

### **3.1.5 Ligation of the 2-cistron fragment into pKK223.3. Production of a 2-cistron vector.**

The vector of choice for the expression of RNase was pKK223.3 (Pharmacia-LKB). This plasmid contains a hybrid promoter, which comprises the “-35” region of the *trp* promoter and the “-10” region of the *lac* promoter, and it has been designated the *tac* promoter. This *tac* promoter also contains the *lac* operator region, therefore the expression of the recombinant protein can be regulated in *E. coli* strains overproducing *lac* repressor. Induction was achieved by addition of the inducer of the *lac* operon, IPTG.

The plasmid pQR162 was digested with *Eco*RI and the products of the digestion were resolved by agarose gel electrophoresis. The DNA fragment corresponding to the 2-cistron fragment was gel extracted and ligated into the expression vector, pKK223.3 previously digested and dephosphorylated (see 2.18). The ligated products were used to transform an aliquot of competent cells (see 2.4 and 2.5). An aliquot of transformed cells (100  $\mu$ l) was spread onto agar plates containing ampicillin (see 2.2) and grown at 37 °C. Random colonies were selected and grown in agar plates containing Ampicillin and also in 5 ml of nutrient broth. Isolation of plasmid DNA was carried out by the method described in 2.8.1. The plasmid DNA thus obtained was digested with *Eco*RI to reveal the insert and *Pst*I to ascertain orientation with respect to the *tac* promoter (see Fig. 3.3). The plasmid containing the 2-cistron fragment in the correct orientation was designated pQR163 (see Fig. 3.1A).

### **3.2 Expression and purification of recombinant RNase in *E.coli* cells harboring the 2-cistron vector, pQR163.**

Several attempts have previously been made to express RNase in *E.coli* either as an intracellular inactive hybrid protein linked to  $\beta$ -galactosidase by a tetrapeptide (Nambiar *et al.*, 1987) or as inclusion bodies containing formylated N-terminus methionine residues (McGeehan and Benner, 1989). The enzyme has also been



expressed using *B.subtilis* as a host and in this system the product is secreted into the medium, with associated problems due to proteolysis (Vasantha and Filpula, 1989).

The production of a clone (pQR163) containing the coding sequence for pre-RNase presents the possibility of obtaining mature RNase in the periplasm of *Escherichia coli*. The signal sequence of RNase has structural features in common with other signal sequences (Gierasch, 1989); a) an amino terminal region with a net positive charge, b) a hydrophobic core, c) seven residues preceding the cleavage site, including proline at position -4 and turn formers, and d) a motif immediately preceding the cleavage site of the form *axa*, being *a*; an amino acid with short side chain (serine at position -3 and glycine at position -1) and *x* being any amino acid (leucine at position -2). (See Fig.3.4). The only difference when compare to prokaryotic signal sequences is that the amino acid at position *a* is an alanine.

The plasmid pQR163 contains two ribosome binding sites, one provided by the *tac* promoter of the vector and the other, which enables translation of the second cistron, is contained within the coding sequence of the first cistron. The mRNA produced upon induction of *E.coli* cells harboring pQR163 with IPTG is bicistronic and starts from the *tac* promoter. The ribosome locates the RBS provided by the vector and the initiation codon of the first cistron, thus a stretch of six amino acids (Met-Phe-Leu-Glu-Asp-Asp) is synthesized (see Fig. 3.4) before the ribosome locates the termination codon of the first cistron. However, before it dissociates from the mRNA, it locates the RBS contained within the first cistron and the initiation codon of the RNase precursor (second cistron) and translation of the mRNA is initiated (see Fig.3.4). As protein synthesis continues, translocation to the periplasm occurs by virtue of the RNase signal sequence. In the process the signal sequence is cleaved as is shown by N-terminus sequencing (see Fig. 3.9). The oxidative environment of the periplasm allows for the correct folding of RNase to form the native enzyme as is evidenced by the recovery of full enzyme activity from the periplasm (see Fig.3.7B).

The catalytic activity of recombinant RNase was assessed by a rate assay involving the hydrolysis of CpA (see section 5.3).

### **3.2.1 Induction of *E.coli* cells harbouring pQR163 with IPTG.**

An aliquot of *E.coli* cells containing pQR163 in 20% glycerol was used to inoculate a specially prepared medium (see 2.2). The culture was left to grow for 4-5 hours at 28 °C with shaking. An aliquot was obtained to assess growth by measuring the optical density at 550 nm. The inducer, IPTG, was added at a final concentration of 0.5 mM when the cells were actively growing ( $OD_{550} = 2$ ). The growth of cells after induction was left to proceed overnight at 28 °C with shaking. Increasing concentrations of IPTG had been used to induce the expression of RNase, however a decrease in yield was obtained when inducing with 2.0 mM IPTG (see Fig. 3.6A). Further work is necessary in order to optimize the expression, this may include varying the length of induced expression and addition of the inducer at different cell densities.

### **3.2.2 Isolation of the periplasmic proteins.**

The release of the periplasmic proteins was carried out using a modified spheroplastic/osmotic shock procedure developed by C. French *et al.*, (unpublished) (see 2.20). This method includes a treatment with lysozyme (500 µg/ml) to fully hydrolyze the outer membrane of *E.coli*. However, for the release of recombinant RNase, the treatment with lysozyme is not necessary. The presence of Na<sub>2</sub>EDTA and Tris/HCl is sufficient for the release of recombinant RNase. Consequently, there are less proteins released and the purification of recombinant RNase by cation exchange chromatography is even more efficient (see sections 4.3.3, 4.4.3 and 4.5.3).

### **3.2.3 Purification of recombinant RNase.**

Purification of recombinant RNase expressed in *E.coli* using the 2-cistron vector (or pQR163) is facilitated by the net positive charge of the protein at a pH near neutrality. Cation exchange chromatography was used to obtain all the positively charged

proteins from the periplasmic extract (see 2.23.1 and Fig. 3.5A). Proteins obtained after isocratic elution were separated by electrophoresis on a 16% polyacrylamide-SDS gel (see 2.22.3) and silver stained (see 2.22.5) (see Fig. 3.6A). Recombinant RNase was purified from the concentrated pool of positively charged proteins by cation exchange chromatography (see 2.23.2 and 2.23.3) (see Fig. 3.5B and 3.5C). Recombinant RNase has been found to elute at the same position as the commercial enzyme (160 mM NaCl). Assessment of the purity of recombinant RNase by polyacrylamide gel electrophoresis and silver staining (see Fig. 3.6A), clearly shows that the strategy followed separates the protein to homogeneity. Chromatographic procedures are achieved in 4 hours.

An experiment was carried out in order to ascertain the percentage distribution of recombinant RNase under the conditions of growth employed since it is possible that some recombinant enzyme leaks to the growth medium from the periplasm and that some is still trapped after the modified spheroplastic/osmotic shock procedure (see 2.20).

Two cultures of 250 ml, one containing JM107 with pKK223.3 and the other containing JM107 with pQR163, were set up. The cultures were induced with 0.5 mM IPTG and growth was left to proceed overnight at 28 °C. The cells from each culture were pelleted and the supernatant (250 ml) was concentrated 125 fold by ethanol precipitation (C. J. Taylorson, personal communication). The pellets were resuspended in buffer and a spheroplastic/osmotic shock procedure was carried out (see 2.20). After centrifugation (see 2.20), the periplasmic fractions were stored on ice, and the pellets sonicated in water to release intracellular proteins including any recombinant RNase not released after the spheroplastic/osmotic shock procedure. After centrifugation (as above) the intracellular fractions were also stored on ice.

All the fractions (supernatant, periplasmic and intracellular) were treated similarly. Each fraction was chromatographed on S-Sepharose FF and all the positively

charged proteins were obtained by isocratic elution (see 2.23.1). The pool of positively charged proteins was concentrated and desalted into water (see 2.23.2). An aliquot from each fraction was electrophoresed on SDS-PAGE (see Fig. 3.6B). Recombinant RNase was not found in the intracellular fraction, indicating that the spheroplastic/osmotic shock procedure was very efficient in releasing the periplasmic contents. However, some leakage of recombinant RNase to the medium was detected (17%) as evidenced by the densitometric measurements of bands on the gel (see 2.27).

This leakage could be explained by the fact that the cells after 20 hours of growth were beginning to lyse and therefore releasing the recombinant RNase into the medium. A possible way to overcome this problem would be to harvest the cells 4 hours after addition of the inducer, thus minimizing cell lysis. However, sufficient recombinant RNase is produced per litre of culture (approx. 100  $\mu\text{g}$ ) to make kinetic characterization possible.

Although the yields are comparatively low (around 100  $\mu\text{g}/\text{l}$  of culture)<sup>compared</sup> to the systems described by McGeehan and Benner, (1989) and Vasantha and Filpula, (1989), it is highly advantageous since there is no need for solubilization of inclusion bodies, refolding of the protein or complicated purification procedures. The total procedure takes just 48 hours from inoculating the medium to obtaining pure and fully active recombinant RNase. The presence of ribonuclease I in the periplasm of *E. coli* (Meador et al., 1990) does not allow the determination of specific activity on the initial periplasmic extract and the reason for variable protein yield is not yet clear. The endogenous *E. coli* ribonuclease I is separated from recombinant RNase on the Mono-S column. Strategies to increase the yield are underway in order to make crystals of the recombinant enzyme and its variants.

**Figure 3.5:** Purification of recombinant bovine pancreatic ribonuclease A (RNase).

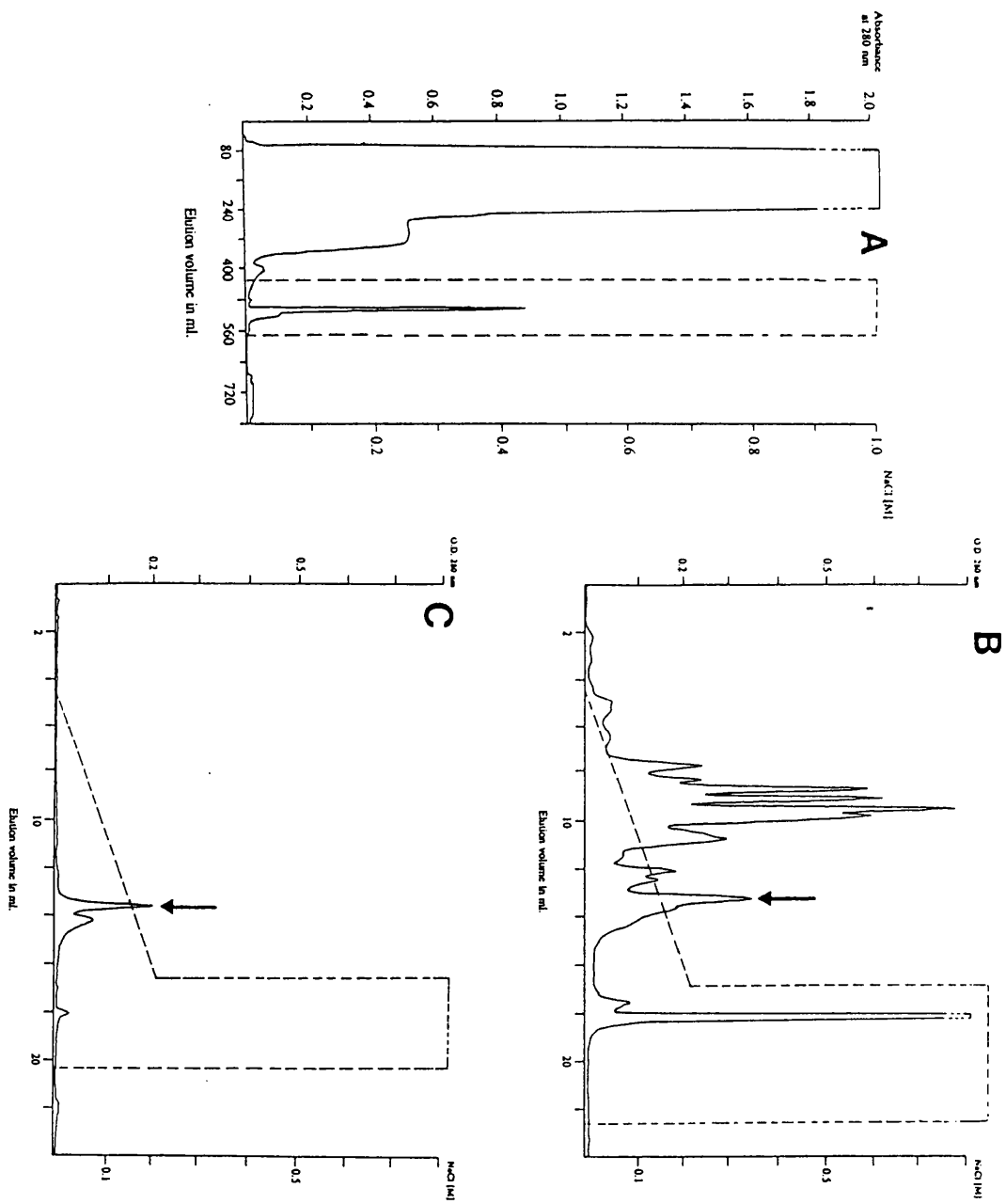
**A.-** Isolation of the positively charged proteins from the periplasmic extract by isocratic elution. The periplasmic extract was filtered through a cellulose nitrate filter (pore size, 0.45  $\mu\text{m}$ ) and loaded onto a column containing S-sepharose (gel bed dimensions: 25 cm x 16 mm) at a flow rate of 4 ml/min. The column was then washed with 50 mM MES pH 6.5 (250 ml) followed by an isocratic elution with 50 mM MES pH 6.5, 1 M NaCl (250 ml) to elute bound proteins. The fractions corresponding to the peak obtained after isocratic elution, which contained positively charged proteins were pooled in a ultrafiltration cell (Amicon, model 8050). Filtration of the sample was carried out through a YM-10 ultrafiltration membrane (M.W. cut off > 10000). The retentate was desalted on a G-25 column (NAP-25, Pharmacia-LKB).

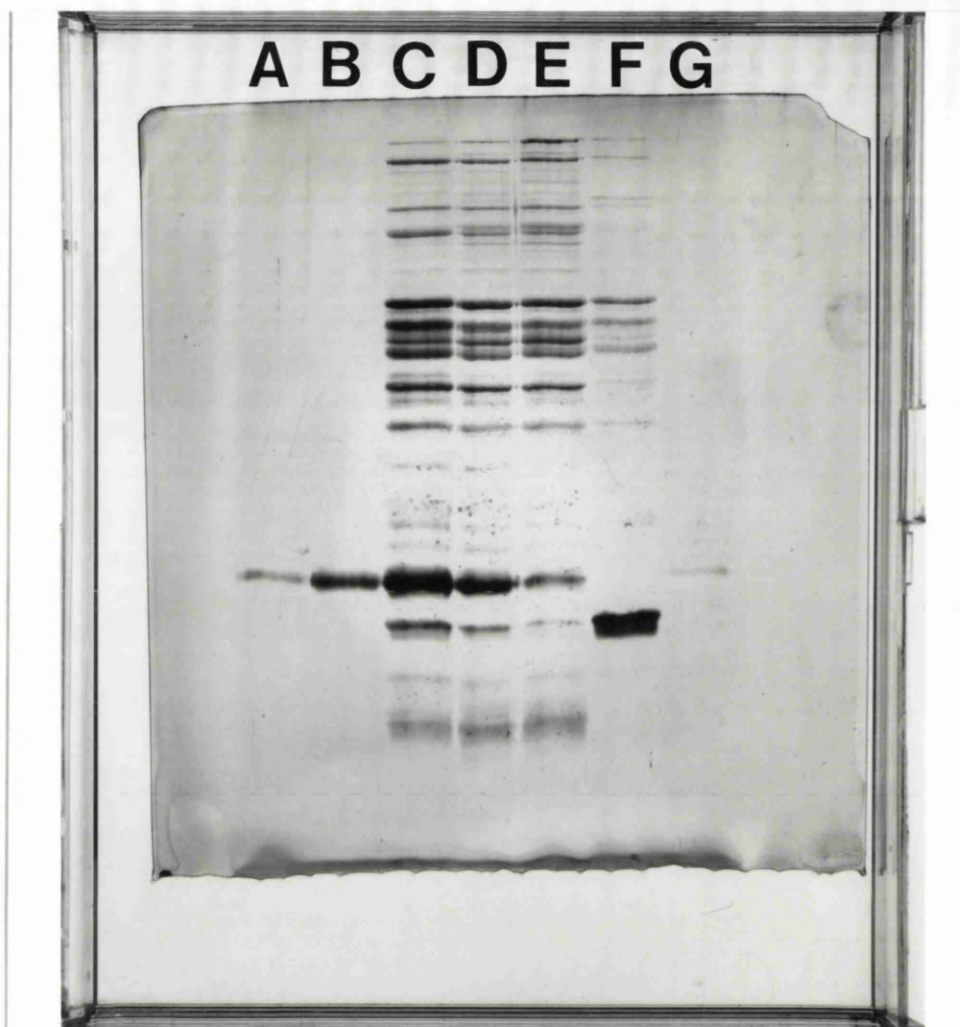
**B.-** Purification of recombinant RNase. The recombinant enzyme was purified from the desalted pool of positively charged proteins by FPLC using a Mono-S column. Elution was carried out using buffer A, 50 mM MES pH 6.5 and buffer, B, 50 mM MES pH 6.5, 1 M NaCl. A gradient was applied of 17.5 mM NaCl/min. at a flow rate of 1 ml/min. The peak obtained at 160 mM NaCl showed RNase activity (arrow). Recombinant RNase was then desalted on a G-25 column (NAP-5, Pharmacia-LKB).

**C.-** Re-run of the peak eluted at 160 mM NaCl in panel **B**, the major peak is recombinant RNase (arrow).

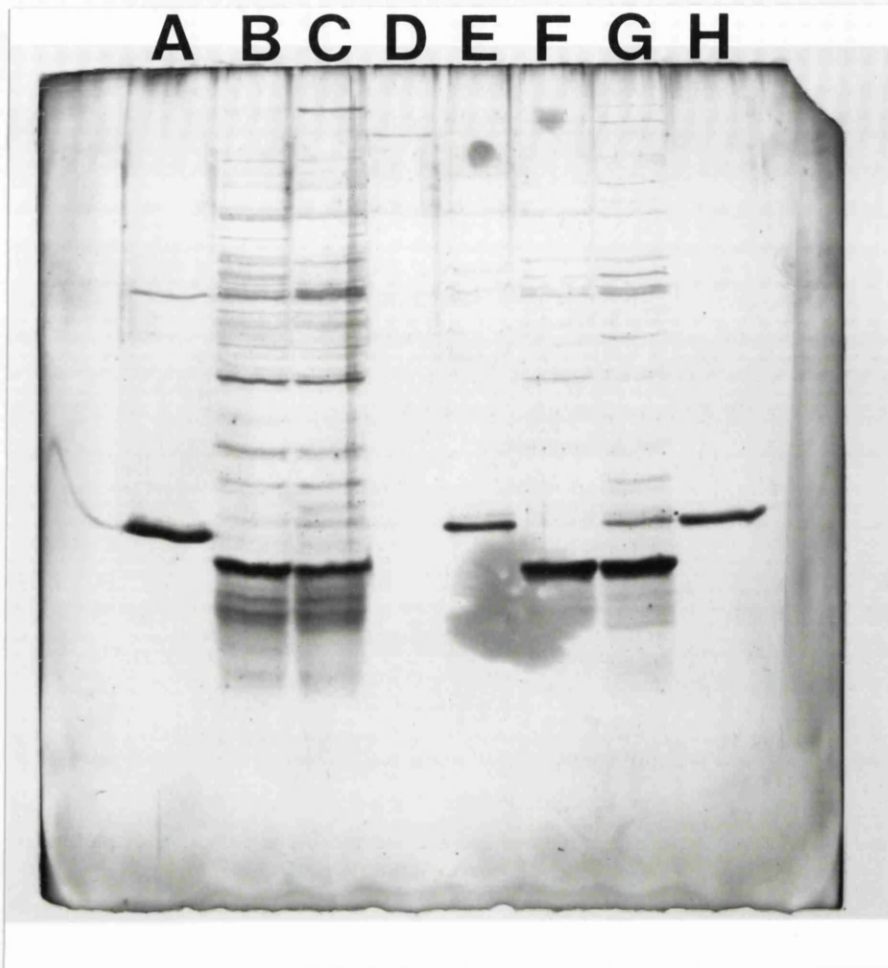
The NaCl gradient is represented by thin dashed lines.

Figure 3.5





**Figure 3.6A:** Assessment of the purity of recombinant RNase on silver stained 16%SDS-PAGE. Lanes **A** and **G** correspond to the commercial enzyme (molecular weight, 13680, 200 ng). Lanes **C-E**, correspond to positively charged proteins (30  $\mu\text{g}$ ) obtained after isocratic elution of the periplasmic extract of *E.coli* cultures harbouring pQR163 and induced with 0.5, 2 and 0 mM IPTG respectively. Lane **F**, as lanes **C-E** but from a culture containing *E.coli* cells harbouring pKK223.3 (control, 30  $\mu\text{g}$ ). Lane **B**, shows the purified recombinant RNase (2  $\mu\text{g}$ ) after ion-exchange chromatography (see Fig. 5.3C).



**Figure 3.6B:** Assessment of leakage of recombinant RNase to the media on a silver stained 14% SDS-PAGE gel. Lane **A**, assessment of the purity of the mutant RNase E111Q ( 1  $\mu\text{g}$ , see section 4.6.3); lane **H**, commercial RNase (1  $\mu\text{g}$ ); lanes **B-G** are; positively charged proteins from the intracellular fraction (**B**<sup>1</sup> and **C**), positively charged proteins from the medium fraction (**D**<sup>1</sup> and **E**) and positively charged proteins from the periplasmic fraction (**F**<sup>1</sup> and **G**).

<sup>1</sup> Fractions obtained from cultures of *E.coli* harbouring the expression vector but without the gene coding for pre-RNase.



### 3.3 A qualitative assay on RNA-agar plates.

In any purification strategy, it is of extreme importance to have the means to monitor the activity of the protein to be purified. In order to accomplish this requirement, an activity assay was developed involving the hydrolysis of RNA, a natural substrate for bovine pancreatic RNase.

RNA-agar plates were prepared as described in section 2.26.1. It is known that *E. coli* has an endogenous RNase (RNase I, Meador et al., 1990), therefore a control experiment was carried out, to see if it was possible to differentiate between bovine pancreatic RNase-expressing colonies and those not producing the enzyme. Four small cultures (5 ml) were inoculated with JM107 (the *E. coli* strain used for expression studies), JM107 with the expression vector pKK223.3 (but without the gene coding for RNase), JM107 with pQR163 (see 3.1.5) and JM107 with pQR173 (an *E. coli* strain expressing a mutated form of RNase, see section 4.6) respectively. The latter three cultures were induced with 0.5 mM IPTG and left to grow with shaking overnight at 37 °C. Cells were then pelleted and an osmotic shock procedure was carried out to release the periplasmic proteins (see 3.2.2). The extract was assayed for RNase activity and the result is shown in Fig. 3.7A.

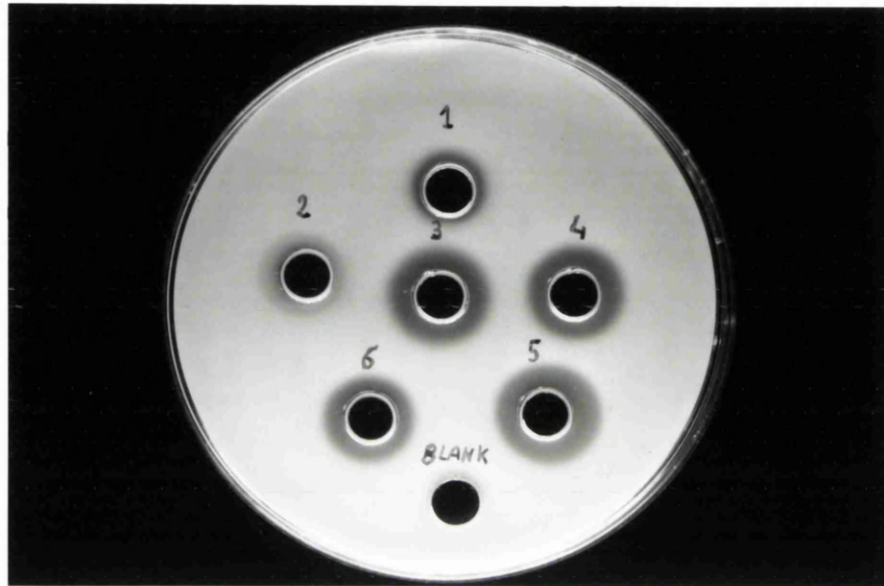
It is clear that prominent zones of activity are only produced around the holes where periplasmic proteins obtained from cultures expressing RNase were present. Small zones of activity can be seen around the holes where periplasmic proteins obtained from JM107 and JM107 harboring the expression vector pKK223.3 (without the gene coding for RNase) were present. This indicates a RNase activity which probably represents RNase I. These results indicate that it is possible to carry out preliminary screens of colonies engineered to produce recombinant RNase prior to purification. This strategy is not, however, of use for the screening of recombinant mutants since a protein may be produced which is not active against RNA. Therefore, the absence of a zone of activity on RNA plates does not necessarily correlate with no expression.

This assay has also been used to determine the activity of the purified recombinant RNase. After determining the concentration of the purified recombinant RNase by the method of Bradford (1976) (see 2.21), an aliquot (0.1 or 0.5  $\mu\text{g}$ ) was tested for activity on a RNA-agar plate (see Fig. 3.7B). From the zone of activity produced, it is observed that recombinant RNase has an equivalent activity to that of commercial ribonuclease.

### **3.4 N-terminus sequencing of recombinant RNase.**

In order to ascertain that the recombinant pre-RNase had been correctly processed to native RNase, the first 20 amino acids of the expressed and purified RNase were sequenced as described in 2.24. The sequence of the purified enzyme was shown to be identical to that of the bovine enzyme indicating correct and efficient removal of the RNase signal sequence during the process of translocation. The first nine aas. are represented in Fig. 3.8.

The expression of RNase using its own signal sequence has proved rapid and efficient (the yields given are sufficient for kinetic studies) and has allowed the rapid assessment of the properties of mutants generated by site-directed mutagenesis ( see Chapters 4 and 5). An expression system has been designed which enables the production of recombinant RNase in a soluble and fully active form. The produced protein is translocated to the periplasm by virtue of its inherent leader sequence. N-terminus sequence has determined that the cleavage of the leader sequence produces mature RNase with the first amino acid being a Lysine. Although the expression system has not been optimized, it produces enough recombinant RNase to carry out kinetic measurement. The purification is extremely straight forward due to the net positive charge that the protein has a neutral pH. This system has the additional advantage that recombinant RNase can be obtained in a soluble, fully active form and purified to homogeneity in 48 hrs. Therefore, this system is validated for the production and characterization of mutant RNases designed by site-directed mutagenesis.



**Figure 3.7A:** Zones of hydrolysis produced in the presence of periplasmic proteins obtained from different cultures.

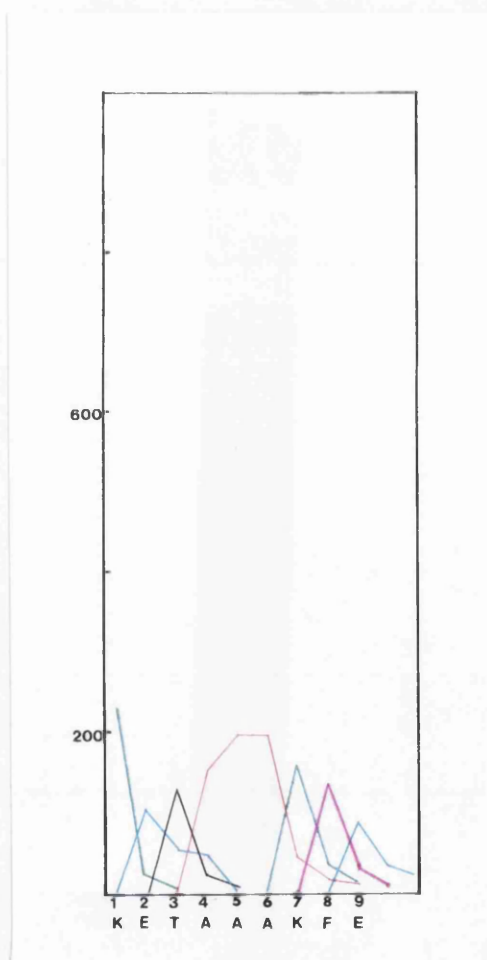
1, periplasmic proteins obtained from *E.coli* JM107 strain; 2, periplasmic proteins obtained from JM107 harbouring pKK223.3; 3, periplasmic proteins obtained from JM107 harbouring pQR163; 4, periplasmic proteins obtained from JM107 harbouring pQR173 (see section 4.6.3); 5 and 6, 2  $\mu\text{g}$  and 0.1  $\mu\text{g}$  of commercial RNase respectively.

Zone size is proportional to the log of the enzyme concentration (Kauker and Jaeger, 1987)



**Figure 3.7B:** Purified recombinant RNase activity on RNA-agar plates.

The top two plates show zones of hydrolysis produced by different amounts of commercial ribonuclease: **a**, 0.1  $\mu\text{g}$ ; **b**, 1  $\mu\text{g}$ ; **c**, 5  $\mu\text{g}$ ; **d**, 25  $\mu\text{g}$ ; **e**, 10  $\mu\text{g}$ ; **g**, control (no enzyme). The bottom plates show the zones of activity or hydrolysis produced by recombinant RNase: **x**, 0.114  $\mu\text{g}$ ; **y**, 0.57  $\mu\text{g}$ ; **z**, an aliquot (10  $\mu\text{l}$ ) of the periplasmic extract obtained after isocratic elution of cells harbouring pQR163 (see Fig. 3.5A).



**Figure 3.8:** N-terminus sequencing of recombinant RNase. Raw yield (pmols) versus residue number.

K, Lysine; E, Glutamic acid; T, Threonine; A, Alanine; F, Phenylalanine.

## CHAPTER 4: SITE-DIRECTED MUTAGENESIS OF BOVINE PANCREATIC RIBONUCLEASE USING THE RECOMBINANT CIRCLE POLYMERASE CHAIN REACTION (RCPCR).

Bovine pancreatic ribonuclease catalyzes the hydrolytic cleavage of ribonucleic acids and 3'-5' linked dinucleotides. One of the unresolved questions concerning RNase action on dinucleotide substrates is the reason why a purine is the preferred base at the B2 subsite rather than a pyrimidine (see Chapter 1). In a series of dinucleotides such as CpA, CpG, CpC and CpU, the measured values of  $k_{cat}$  vary enormously but there are only small differences in the reported values of  $K_m$  (see Chapter 1) (Witzel and Barnard, 1962). This interesting finding indicates that the nature of the leaving group and its interaction with the enzyme are important. Thus, substitutions of amino acids in the vicinity of the leaving group using site-directed mutagenesis should give us an insight into the factors defining the specificity of the enzyme.

The structural information available from X-ray crystallography of RNase-ligand complexes has involved the use of substrate analogues or inhibitors like C-2':5'-A or C-2':5'-G. Wodak *et al.*, (1977) has speculated that the adenine ring interacts with the enzyme as follows: a) the N6 of the adenine ring donates a proton to Asn-71 and to Gln-69, and b) the N1 of the adenine ring interacts with Glu-111 (see Fig. 4.1). This speculation was based on data obtained at 2 Å resolution of complexes produced by soaking a pre-grown crystal of RNase in a solution containing the substrate analogue (Wodak *et al.*, 1977).

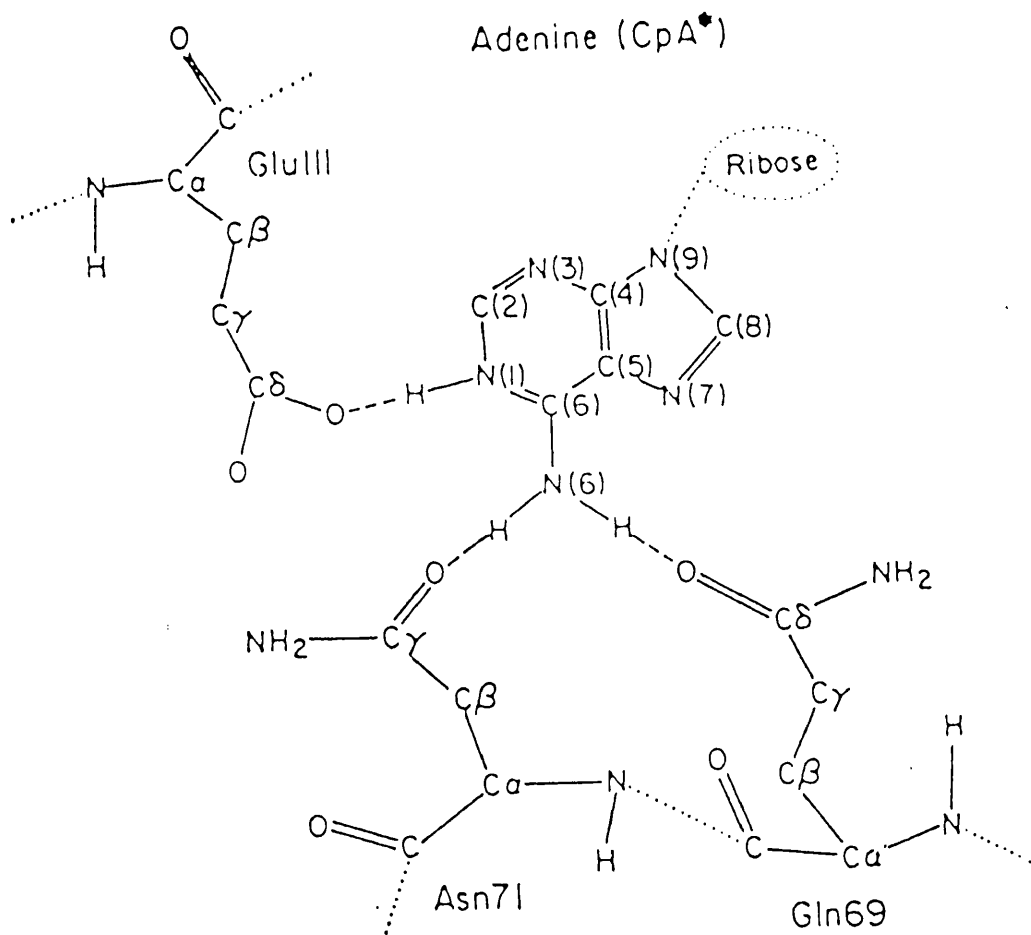
Perry (1987), using data obtained at 2 Å resolution, indicated that the guanine ring could interact with the enzyme to produce either one of two binding modes. In the first (position A, Fig. 4.2) the interactions are similar to those of the Adenine ring: a) The O6 of the guanine ring accepts a proton from Asn-71 and from Gln-69, b) the N1 of the guanine ring is protonated when binding of the substrate is taking place and

interaction occurs with Glu-111, and c) Gln-69 may interact as well with N7 of the guanine ring (Perry, 1987) (see Fig. 4.2).

A recent study by Aguilar *et al.*, (1991) using the same substrate analogue (C-2':5'-G) working at 1.5 Å resolution, has however indicated that the guanine ring could not possibly interact with the B2 subsite. This group has proposed that the guanine ring interacts with the B1 subsite (see Chapter 1) in a retro-binding mode, with the rest of the molecule directed towards the solvent. The method of crystallization again involved the technique of soaking the pre-grown crystal of RNase in a solution containing the inhibitor or substrate analog.

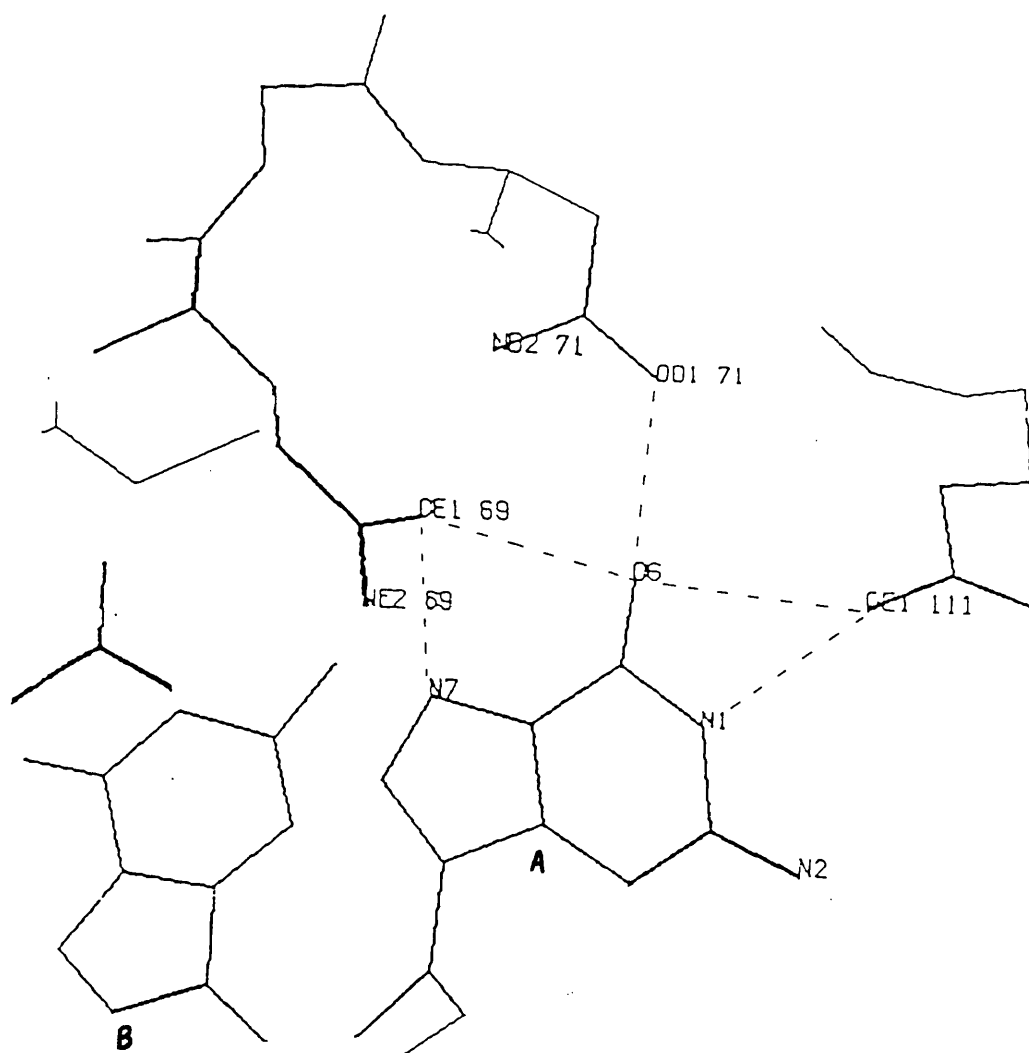
It is clear from this conflicting evidence that the use of substrate analogues which produce non-productive binding can never properly simulate the situation that pertains when productive binding occurs, although useful insight may be gained. Available data have indicated that residues Gln-69, Asn-71 and Glu-111 are implicated in the specificity of RNase. This has been investigated using site-directed mutagenesis using the technique of the recombinant circle polymerase chain reaction (RCPCR). The kinetic properties of mutants thus obtained are described in Chapter 5.

**Figure 4.1:** Interaction of the Adenine ring of the complex C-2':5'-A (CpA\*) with RNase.





**Figure 4.2:** Interaction of the Guanine ring of the complex C-2':5'-G with RNase



#### **4.1 Recombinant Circle Polymerase Chain Reaction (RCPCR).**

The expression system described in Chapter 3 has been designed to produce recombinant RNase in a soluble and fully active form in as little as 48 hours, and in sufficient quantities to perform kinetic analysis. The production of mutant ribonucleases to be expressed in this system has been achieved using an application of the PCR technique.

A rapid and efficient method with as few as possible manipulations was required and RCPCR meets these criteria very well (Jones and Howard, 1990). RCPCR is an “easy to use” technique whereby mutations can be incorporated into the coding sequence for RNase using the polymerase chain reaction. It involves very few steps and colonies expressing mutant RNase can be obtained in 12-15 days.

##### **4.1.1 How does it work?**

RCPCR is based on two PCR incubations. In each of these, a primer is used which has an altered nucleotide sequence coding for the engineered mutation to be incorporated in the expressed recombinant RNase (see Fig. 4.3). The primers for RCPCR are designed such that when used in PCR incubations, the products generated are double stranded DNA fragments with cohesive ends that upon combination, denaturation and annealing produce “nicked” or “gapped” circles ready to transfect (see Fig. 4.3). The size of the gap is directly related to the distance between the two 5' ends of each pair of primers used in the PCR incubations.

The template for all the PCR incubations is pQR162 (see Fig. 3.1A). The parameters of temperatures and cycle times for denaturation, annealing, and extension as well as the number of cycles may vary depending on the type of polymerase used (polymerases with proof-reading activity are slower than those lacking it).

Once the PCR incubations are complete, the PCR products are resolved by agarose gel electrophoresis (see Fig. 4.4). The DNA fragments of interest are gel extracted

(see 2.17) and ethanol precipitated (see 2.7). The pellets are resuspended in a small volume and combined in annealing buffer (see 2.25.2.1). After denaturation for 5 min. at 92 °C, the separated single stranded molecules are left to re-anneal for 2 hours at 55-57 °C. The “nicked” or “gapped” circles are used to transform an aliquot of competent cells (see 2.4 and 2.5 and Fig. 4.3). During the time of annealing it is assumed that a higher proportion of molecules re-anneal forming linear double stranded DNA fragments and only a small proportion form “nicked” or “gapped” circles. This is an assumption based on the observation that the transformation frequency is low.

A small number of colonies (2-4) are grown up in 5 ml of nutrient broth overnight at 37 °C. The plasmid content of the recombinant colonies is then isolated and used as template for double stranded DNA sequencing. The data obtained from sequencing gels revealed that a high number of colonies containing the parental template (pQR162) decreases considerably when smaller amount of template is used. This was found to be due to carry over of template DNA when fragments were gel extracted. The template DNA contaminating the extracted product is supercoiled and consequently has a high transformation frequency. However, carry over can be significantly reduced by lowering the amount of template DNA used.

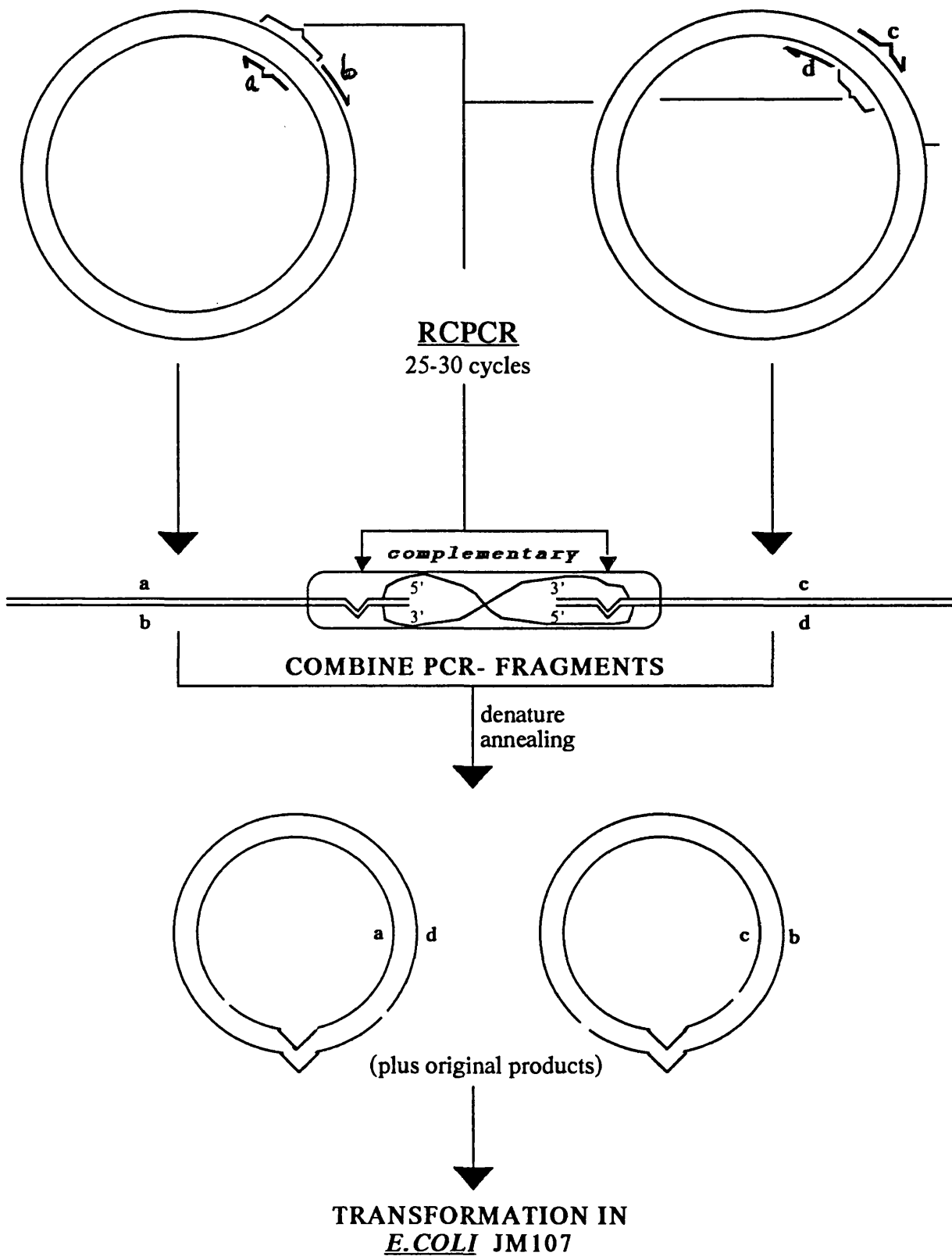
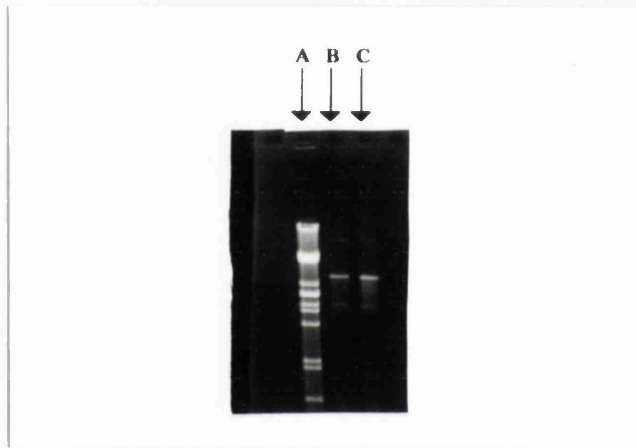


Figure 4.3: Site-directed mutagenesis using RCPCR



**Figure 4.4:** PCR products resolved by agarose gel electrophoresis. Lane A, Lambda DNA digested with *Pst*I; lane B, PCR fragment produced by using primers 60 and 80 (**a** and **b** in Fig. 4.3); lane C, PCR fragment produced by using primers 70 and 90 (**c** and **d** Fig. 4.3). The size of the PCR fragment is around 3.2 Kb.

#### **4.1.2 Advantages and disadvantages.**

The advantage of using the RCPCR technique is that colonies harbouring a plasmid containing the altered coding sequence for bovine pancreatic RNase can be obtained in less than two days. The manipulations, reduced to a minimum, are: 2 PCR incubations, electrophoresis and transformation.

All the mutations carried out in this work have been performed using RCPCR. The gapped circles obtained, after combining the PCR products, contained gaps of 0-9 nucleotides. Double stranded DNA sequencing of the products revealed that these gapped circles were repaired on transformation into competent cells of *E. coli*.

RCPCR has further potential by the use of larger gaps of 50-200 nucleotides. In this system, two primers are synthesized complementary to the 5' and 3' ends of the coding sequence of pre-RNase (constant primers) and only 2 primers will be required each time for any mutation that is thought of. However, larger gaps may present a problem for the *E.coli* repair mechanisms and in this case an extra step involving the addition of the Klenow fragment and nucleotides to fill-in the gap just before transformation would be needed.

One of the problems associated with the technique of PCR, is the misincorporation frequency of the polymerase used. Enzymes are available however, which possess proof-reading activity and the use of these enzymes is recommended.

#### **4.2 Production of the mutant RNase K41R.**

Lysine-41 is a key amino acid involved in the hydrolysis of ribonucleic acid and dinucleotides. Its function is not clear, but evidence suggests an involvement in the stabilization of some of the intermediates of the reaction pathway (see Chapter 1).

This mutation has already been carried out utilizing other mutagenesis techniques (Trautwein *et al.*, 1991), and therefore this substitution of lysine for arginine was used as a control to test the technique of RCPCR.

#### 4.2.1 Primers for RCPCR.

Four primers were synthesized and purified (see 2.13.4.1). They were designated primers; 60, 70, 80 and 90 (See Fig.4.5).

Figure 4.5: Nucleotide sequence of the primers used in RCPCR to produce the mutant RNase K41R.

Primer 60: 5' -AAGATCGATGCC**CGT**CCAGTGAACACCTTTG -3'

Primer 70: 5' -GTGTTCACTGG**ACG**GCATCGATCTTTGGTC -3'

Primer 80: 5' -TGGTCAGGTTCCGGCTCTTCATCATCTGGT -3'

Primer 90: 5' -CTTTGTGCACGAGTCCCTGGCTGATGTCCA -3'

**CGT**: Codon for Arg at position 41 of RNase.

**ACG**: Complementary sequence.

Two PCR incubations were set up using pQR162 as template (500 ng). In each of the PCR incubations, one of the primers contained the altered nucleotide sequence corresponding to amino acid 41. (see Fig.4.3). One incubation contained primers 60 and 80 (a and b in Fig. 4.3) and the other contained primers 70 and 90 (c and d in Fig. 4.3).

#### 4.2.2 Conditions for the PCR incubations.

The PCR incubations were carried out for 25 cycles, each comprising denaturation for 2 min. at 92 °C, annealing for 2 min. at 55 °C and extension for 5 min. at 72 °C (see 2.25.2.1). The products of the two PCR incubations were resolved by agarose gel electrophoresis (see Fig. 4.4). The DNA fragments of interest (approx. 3.2 Kb) were

gel extracted (see 2.17) and ethanol precipitated (see 2.7). Both PCR fragments were combined in annealing buffer (see 2.25.2.1) and after denaturation for 5 min. at 92 °C were left to re-anneal for 2 hours at 55 °C. The re-annealed products were then used to transform an aliquot of competent cells (see 2.4 and 2.5).

Two colonies were randomly selected and further grown in 5 ml. of nutrient broth overnight at 37 °C with shaking. The plasmid content was isolated (see 2.8.1) and double stranded DNA sequencing was carried out (see 2.16). The DNA sequence revealed that one of the recombinant plasmids contained the incorporated mutation. This construct was named pQR164.

pQR164 was then digested with *EcoRI* (see 2.10) and the products of the digestion resolved by agarose gel electrophoresis. The 2-cistron fragment containing the codon for arginine at position 41 of RNase was gel extracted (see 2.17) and ligated into pKK223.3 (see 2.18) forming a construct designated pQR165.

#### **4.2.3 Production and purification of the mutant RNase K41R.**

*E. coli* JM107 colonies harboring pQR165 were grown in 5 litres of a specially prepared medium (see 2.2). When the cell density reached an OD<sub>550nm</sub> of 2.0, the culture was induced with IPTG. Growth was continued overnight at 28 °C with shaking. The proteins contained in the periplasm were released by a spheroplastic/osmotic shock procedure (see 2.20). The contents were loaded onto a S-Sepharose FF column and isocratic elution was carried out to isolate the positively charged proteins. The recombinant RNase K41R mutant was resolved from the pool of positively charged proteins by cation exchange chromatography (see 2.23 and Fig. 4.6). The yield obtained was higher than that obtained when expressing recombinant RNase, the reasons for this is not known. The mutant appears pure by virtue of the fact that it runs as a single band on a silver stained SDS-PAGE gel (see Fig. 4.14, lane A).



#### **4.2.4 Effect of the mutation.**

An aliquot of the recombinant mutant (1  $\mu\text{g}$ ) was tested for RNase activity on RNA-agar plates (see 2.26.1), the halo of activity revealed after the addition of 2 M HCl was markedly smaller than that produced by the commercial or recombinant enzyme (see Fig. 4.7). The behaviour of the RNase K41R mutant using CpA, CpG, CpC and CpU is described in Chapter 5.

**Figure 4.6:** Purification of the mutant RNase K41R (arrow).

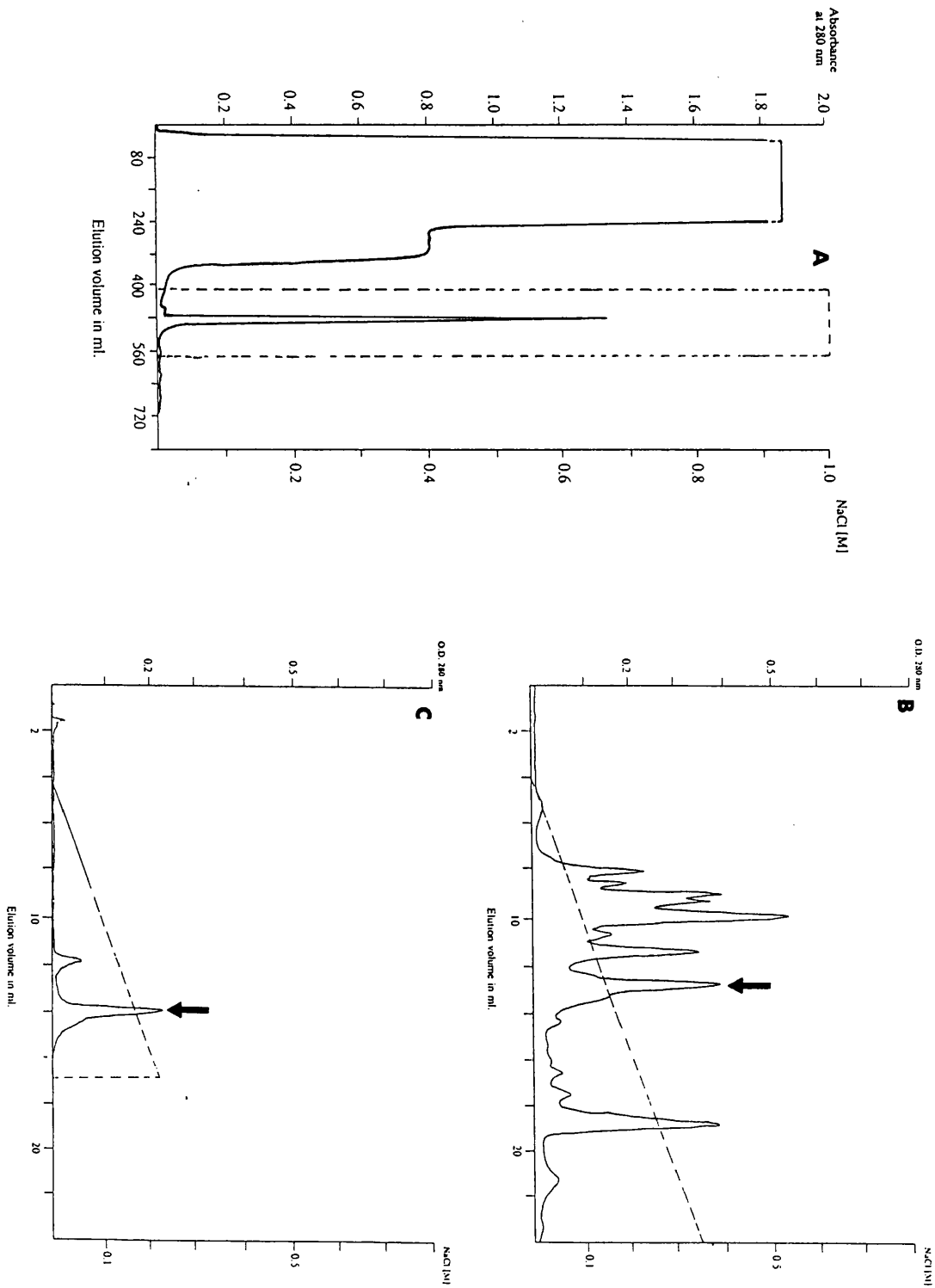
The procedure for purifying this mutant has been carried out in the same way as when purifying recombinant RNase (see legend of figure 3.5).

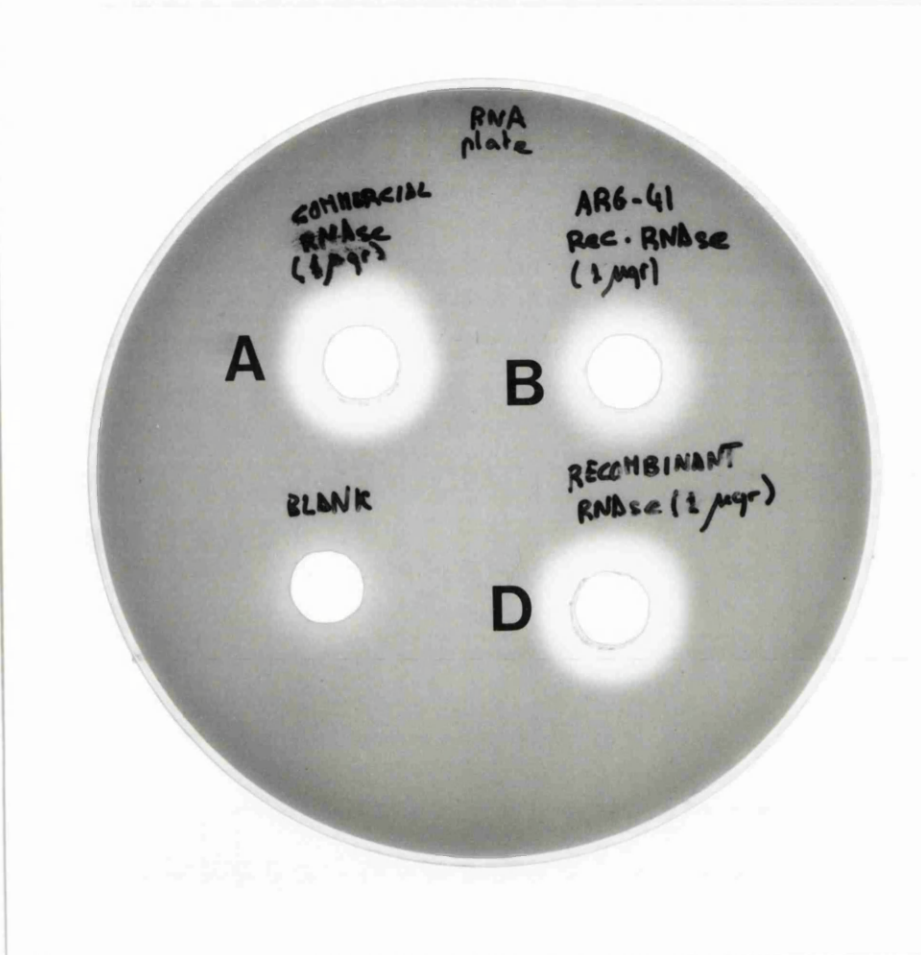
**A.-** Isocratic elution for isolation of all the positively charged proteins from the periplasm of cultures of *Escherichia coli* harbouring pQR 165.

**B.-** Elution profile for the isolation of the mutant RNase K41R (arrow)

**C.-** Final purification of the peak obtained in **B.**

Figure 4.6





**Figure 4.7:** Activity on RNA-agar plates of the mutant RNase K41R.

**A** and **D**, zones of activity produced by 1  $\mu\text{g}$  of commercial and recombinant RNase respectively. **B**, zone of activity produced by the mutant RNase K41R (1  $\mu\text{g}$ ).

### 4.3 Production of the mutant RNase Q69A.

The following sections described mutations that change the amino acids surrounding the leaving group (see Figs. 4.1 and 4.2). These are Q69A (this section), N71A (see 4.4), a double mutant (see 4.5) and E111Q (see 4.6).

#### 4.3.1 Primers for RCPCR.

Four primers were synthesized and purified (see 2.13.4.1). They were designated primers; 100, 101, 102 and 103 (see Fig.4.8).

Figure 4.8: Nucleotide sequence of the primers used in RCPCR to produce the mutant RNase Q69A.

Primer 100: 5' -TGCAAGAATGGGG**GCT**ACCAATTGCTACCAG -3'

Primer 101: 5' -GGCAACATTTTTCTGGGAGCACACGGCCTG -3'

Primer 102: 5' -GTAGCAATTGGT**AGC**CCCATTCCTGCAGGC- 3'

Primer 103: 5' -TCCACCATGAGCATCACCGACTGCCGTGAG- 3'

**GCT**: Codon for Ala at position 69 of RNase.

**AGC**: Complementary sequence.

Two PCR incubations were set up using pQR162 as template (20 ng). In each of the PCR incubations, one of the primer contained the altered nucleotide sequence corresponding to amino acid 69 (see Fig.4.3). One incubation contained primers 100 and 101 (**a** and **b** in Fig. 4.3) and the other contained primers 102 and 103 (**c** and **d** in Fig. 4.3). The versatility of RCPCR enables the distance between the two 5' ends in each reaction to be varied. In this case, there was no gap between the two 5' ends of primers 100 and 101, but the distance between the 5' ends of primers 102 and 103 was 9 nucleotides.

### 4.3.2 Conditions for the PCR incubations.

The PCR incubations were carried out for 25 cycles, each comprising denaturation for 1.5 min. at 92 °C, annealing for 1 min. at 57 °C and extension for 4 min. at 75 °C (see 2.25.2.2). The products of the PCR incubations were treated as in section 4.2.2 but the annealing temperature used for the combined fragments was 57 °C. The re-annealed products were then used to transform an aliquot of competent cells (see 2.4 and 2.5).

Four colonies were randomly selected and further grown in 5 ml. of nutrient broth overnight at 37 °C with shaking. The plasmid content was isolated (see 2.8.1) and double stranded DNA sequencing was carried out (see 2.16). The DNA sequence revealed that one of the 4 of the recombinant plasmids had the mutation incorporated. This construct was named pQR166. The 2-cistron fragment of pQR166 was subcloned into the expression vector as described in section 4.2.2. The new construct was designated pQR167.

### 4.3.3 Production and purification of the mutant RNase Q69A.

*E.coli* JM107 colonies harbouring pQR167 were grown in 5 litres of a specially prepared medium (see 2.2). The culture was induced as described in section 4.2.3. After harvesting, the cells were osmotically shocked as described in section 2.20 to release periplasmic proteins. The periplasmic extract was processed as described in 2.23. The elution profiles for the purification of the mutant enzyme are shown in Fig. 4.9. The yields of the mutant were similar to those obtained for recombinant RNase (100 µg/l of culture). This indicates that for the release of mutant RNase from the periplasm, an osmotic shock with Tris-EDTA is sufficient (see 2.20). The omission of the lysozyme from this procedure makes purification of the mutant RNase easier and improves resolution (see Fig.4.9). The mutant appears pure by virtue of the fact that it runs as a single band on a silver stained SDS-PAGE gel (see Fig. 4.14, lane B).

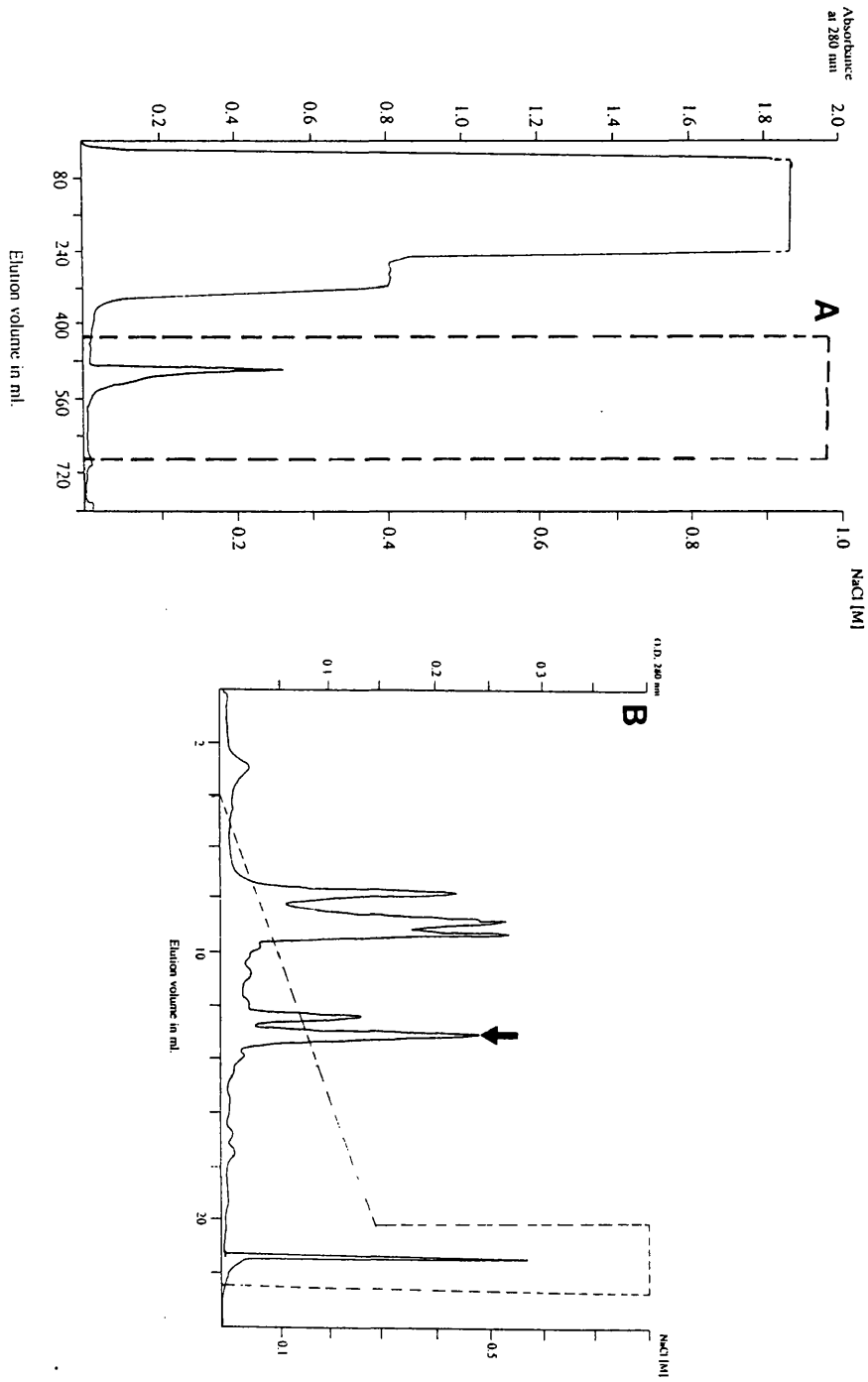
**Figure 4.9:** Purification of the mutant RNase Q69A (arrow).

The procedure for purifying this mutant has been carried out in the same way as when purifying recombinant RNase (see legend of figure 3.5).

**A.-** Isocratic elution for isolation of all the positively charged proteins from the periplasm of cultures of *Escherichia coli* harbouring pQR 167.

**B.-** Elution profile for the isolation of the mutant RNase Q69A (arrow)

Figure 4.9





#### 4.4 Production of the mutant RNase N71A.

##### 4.4.1 Primers for RCPCR

The fact that the distance between the two 5' ends can be variable means that the 2 external primers (101 and 103, **b** and **d** in Fig. 4.3) can be maintained for the production of the N71A mutant and only two primers need be synthesized and purified (see 2.13.4.1). These primers were designated primers 104 and 105 (see Fig.4.10).

Figure 4.10: Nucleotide sequence of the primers used in RCPCR to produce the mutant RNase N71A.

Primer 104: 5' -AATGGGCAGACCGCTTGCTACCAGAGCTAC- 3'

Primer 105: 5' -GCTCTGGTAGCAAGCGGTCTGCCCATTTCTT -3'

**GCT**: Codon for Ala at position 71 of RNase.

**AGC**: Complementary sequence.

Two PCR incubations were set up using pQR162 as template (2 ng). In each of the PCR incubations, one of the primers contained the altered nucleotide sequence corresponding to amino acid 71 (see Fig.4.3). One incubation contained primers 104 and 101 (**a** and **b** in Fig. 4.3) and the other contained primers 105 and 103 (**c** and **d** in Fig. 4.3). The distance between the two 5' ends of primers 104 and 101 was of 6 nucleotides and that between the 5' ends of primers 105 and 103 was of 3 nucleotides.

##### 4.4.2 Conditions for the PCR incubations.

The PCR incubations were carried out for 30 cycles, each comprising denaturation for 1.5 min. at 92 °C, annealing for 1.5 min. at 56 °C and extension for 6 min. at 75 °C (see 2.25.2.3). The products of the PCR incubations were treated as in section 4.2.2 but the annealing temperature used for the combined fragments was 55 °C. The

re-annealed products were then used to transform an aliquot of competent cells (see 2.4 and 2.5).

Four colonies were randomly selected and further grown in 5 ml. of nutrient broth overnight at 37 °C with shaking. The plasmid content was isolated (see 2.8.1) and double stranded DNA sequencing was carried out (see 2.16). The DNA sequence revealed that all of the recombinant plasmids had the mutation incorporated. This construct was named pQR170. The 2-cistron fragment of pQR170 was subcloned into the expression vector as described in section 4.2.2. The new construct was designated pQR171.

#### **4.4.3 Production and purification of the mutant RNase N71A.**

*E.coli* JM107 colonies harbouring pQR171 were grown in 5 litres of a specially prepared medium (see 2.2). The culture was induced as described in section 4.2.3. After harvesting, the cells were osmotically shocked as described in section 4.3.3 to release periplasmic proteins. The periplasmic extract was processed as described in section 2.23. Elution profiles for the purification of the mutant enzyme are shown in Fig. 4.11. The yields of the mutant enzyme were similar to those obtained for recombinant RNase (100 µg/l of culture), and the mutant has been purified to homogeneity as evidenced by silver staining SDS-PAGE gels (see Fig. 4.14, lane C)

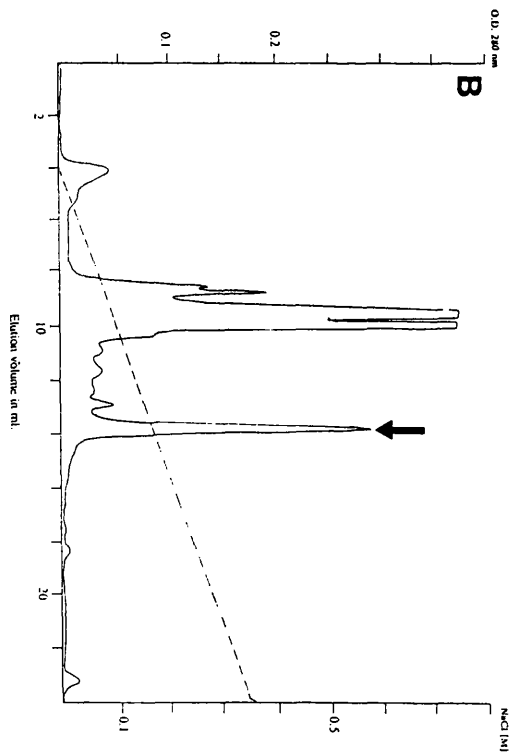
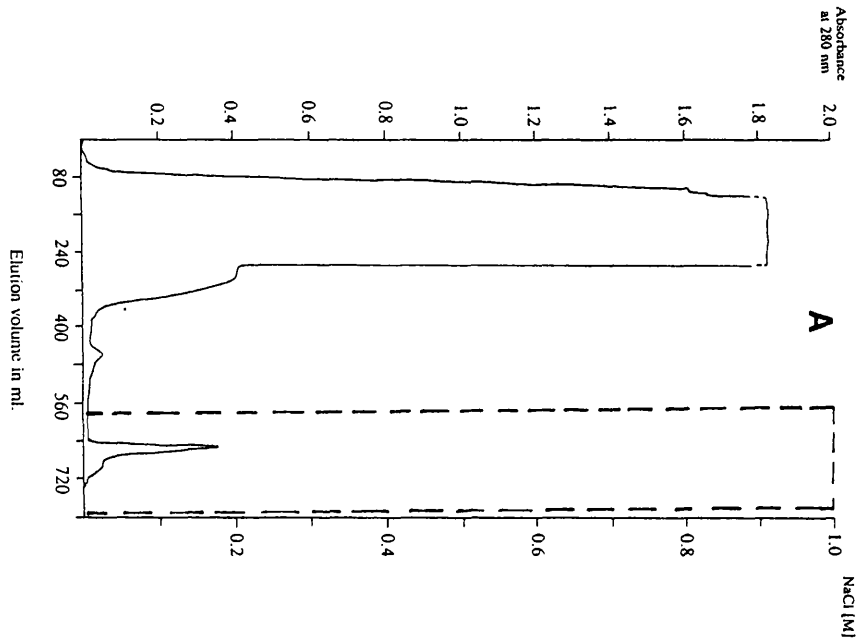
**Figure 4.11: Purification of the mutant RNase N71A (arrow).**

The procedure for purifying this mutant has been carried out in the same way as when purifying recombinant RNase (see legend of figure 3.5).

**A.-** Isocratic elution for isolation of all the positively charged proteins from the periplasm of cultures of *Escherichia coli* harbouring pQR 171.

**B.-** Elution profile for the isolation of the mutant RNase N71A (arrow)

Figure 4.11



## **4.5 Production of the mutant RNase Q69A:N71A.**

### **4.5.1 Primers for RCPCR**

As described in section 4.4.1 it was only necessary to synthesize and purify two primers (see 2.13.4.1). They were designated primers 106 and 107 (see Fig.4.12).

Figure 4.12: Nucleotide sequence of the primers used in RCPCR to engineer the mutant RNase Q69A:N71A .

Primer 106: 5' -TGCAAGAATGGGGCTACCGCTTGCTACCAGAGCTAC -3'

Primer 107: 5' -GCTCTGGTAGCAAGCGGTAGCCCCATTCTTGCAGGC -3'

**GCT:** Codons for Ala at position 69 and 71 of RNase.

**AGC:** Complementary sequences.

Two PCR incubations were set up using pQR162 as template (2 ng). In each of the PCR incubations, one of the primers contained the altered nucleotide sequence corresponding to amino acids 69 and 71 (see Fig.4.3). One incubation contained primers 106 and 101 (a and b in Fig. 4.3) and the other contained primers 107 and 103 (c and d in Fig. 4.3). There was no gap between the two 5' ends of primers 106 and 101 and the distance between the 5' ends of primers 107 and 103 was of 3 nucleotides.

### **4.5.2 Conditions for the PCR incubations.**

PCR incubations were carried out as described in section 4.4.2. Four colonies were randomly selected and further grown in 5 ml. of nutrient broth overnight at 37 °C with shaking. The plasmid content was isolated (see 2.8.1) and double stranded DNA sequencing was carried out (see 2.16). The DNA sequence revealed that all of the recombinant plasmids contained the incorporated mutation. This construct was named pQR168. The 2-cistron fragment of pQR168 was subcloned into the expression vector as described in section 4.2.2. The new construct was designated pQR169.

### 4.5.3 Production and purification of the mutant RNase Q69A:N71A .

*E.coli* JM107 colonies harbouring pQR169 were grown in 5 litres of a specially prepared medium (see 2.2). The culture was induced as described in 4.2.3. After harvesting the cells were osmotically shocked as described in section 4.3.3 to release periplasmic proteins. The periplasmic extract was processed as described in section 2.23. The elution profiles of the purification of the mutant enzyme are shown in Fig. 4.13. The yields of this mutant were however lower to those obtained for recombinant RNase (50  $\mu$ g/l of culture). Silver stained SDS-PAGE gels revealed that the protein obtained has been purified to homogeneity (see Fig. 4.14, lane D).

**Figure 4.13:** Purification of the mutant RNase Q69A:N71A (arrow).

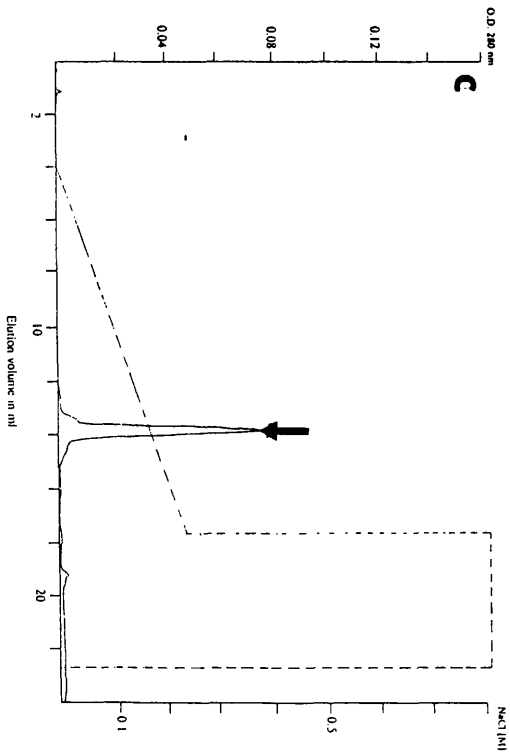
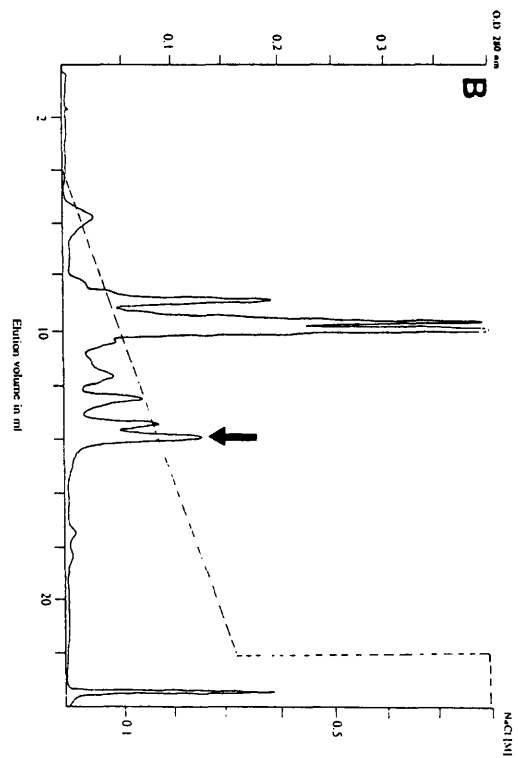
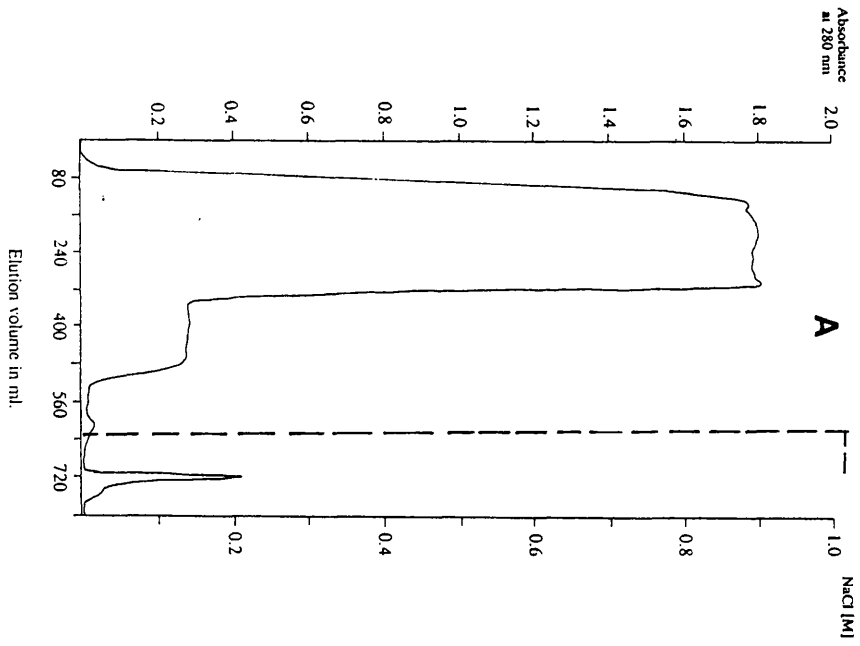
The procedure for purifying this mutant has been carried out in the same way as when purifying recombinant RNase (see legend of figure 3.5).

**A.-** Isocratic elution for isolation of all the positively charged proteins from the periplasm of cultures of *Escherichia coli* harbouring pQR 169.

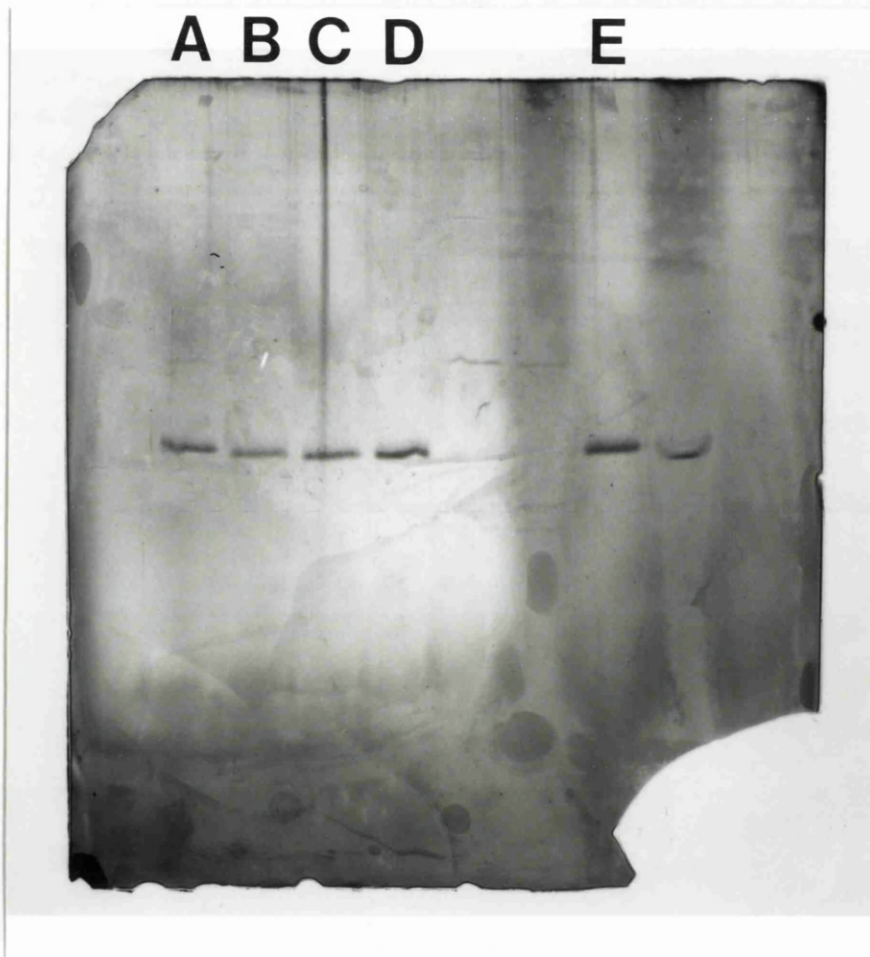
**B.-** Elution profile for the isolation of the mutant RNase Q69A:N71A (arrow).

**C.-** Final purification of the peak eluted in B.

Figure 4.13







**Figure 4.14:** Assessment of the purity of the recombinant mutants on a silver stained 16% SDS-PAGE gel.

Lane A, RNase K41R; lane B, RNase Q69A; lane C, RNase N71A; lane D, RNase Q69A:N71A; lane E, commercial RNase.

In each track, 1  $\mu\text{g}$  of protein was loaded.

## **4.6 Production of the mutant RNase E111Q mutant.**

### **4.6.1 Primers for RCPCR.**

Four primers were synthesized and purified (see 2.13.4.1). They were designated primers; 120, 121, 122 and 123 (see Fig.4.15).

Figure 4.15: Nucleotide sequence of the primers used in RCPCR to engineer the mutant RNase E111Q.

Primer 120: 5' -ATTGTGGCTTGT**CAG**GGAAACCCGTACGTG -3'

Primer 121: 5' -GTGTTTATTCGCCTGGGTGGTCTTGTAGGC -3'

Primer 122: 5' -GTACGGGTTTCC**CTG**ACAAGCCACAATGAT -3'

Primer 123: 5' -CCAGTCCACTTTGATGCTTCAGTGTAGGTC -3'

**CAG:** Codon for Gln at position 111 of RNase.

**CTG:** Complementary sequence.

Two PCR incubations were set up using pQR162 as template (2 ng). In each of the PCR incubations, one of the primers contained the altered nucleotide sequence corresponding to amino acid 111 (see Fig.4.3). One incubation contained primers 120 and 121 (**a** and **b** in Fig. 4.3) and the other contained primers 122 and 123 (**c** and **d** in Fig. 4.3). The distance between the two 5' ends of primers 120 and 121 was 3 nucleotides and that between the 5' ends of primers 122 and 123 was also 3 nucleotides.

### **4.6.2 Conditions for the PCR incubations.**

This was carried out as described in section 4.4.2. Four colonies were randomly selected and further grown in 5 ml. of nutrient broth overnight at 37 °C with shaking. The plasmid content was isolated (see 2.8.1) and double stranded DNA sequencing was carried out (see 2.16). The DNA sequence revealed that all of the recombinant plasmids contained the incorporated mutation. This construct was named pQR172.

The 2-cistron fragment of pQR172 was subcloned into the expression vector as described in section 4.2.2. The new construct was designated pQR173.

#### **4.6.3 Production and purification of the mutant RNase E111Q.**

*E.coli* JM107 colonies harbouring pQR173 were grown in 5 litres of a specially prepared medium (see 2.2). The culture was induced as described in section 4.2.3. After harvesting the cells were osmotically shocked as described in section 4.3.3 to release periplasmic proteins. The periplasmic extract was processed as described in section 2.23. Elution profiles for the purification of the mutant enzyme are shown in Fig. 4.16. The purification of the RNase E111Q mutant was different from those described previously in that the protein was found to elute later (at 210 mM NaCl). This is due to the fact that an amino acid with a negative charge has been replaced by a neutral amino acid. The yield of 50  $\mu\text{g/l}$  of culture was lower than that obtained for recombinant RNase (100  $\mu\text{g/l}$  of culture of culture). A silver stained SDS-PAGE gel showed that the major band purified was mutant RNase, however a very faint band of higher molecular weight was noticed on the gel (see Fig. 3.6B).

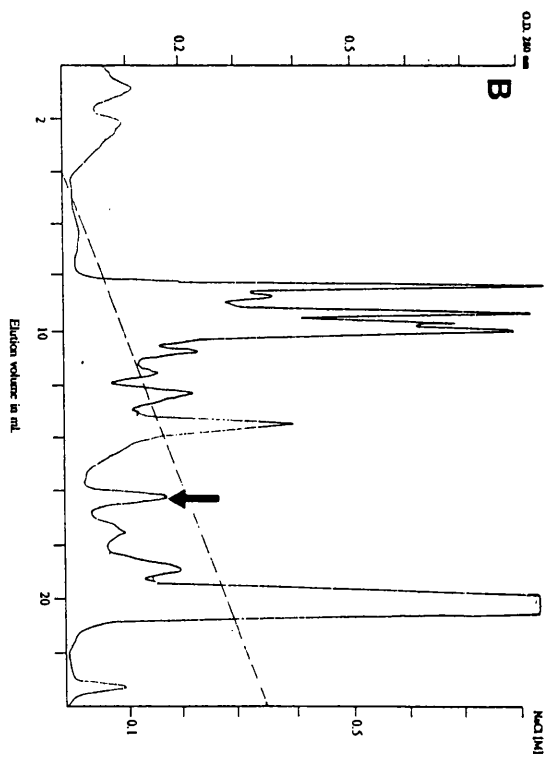
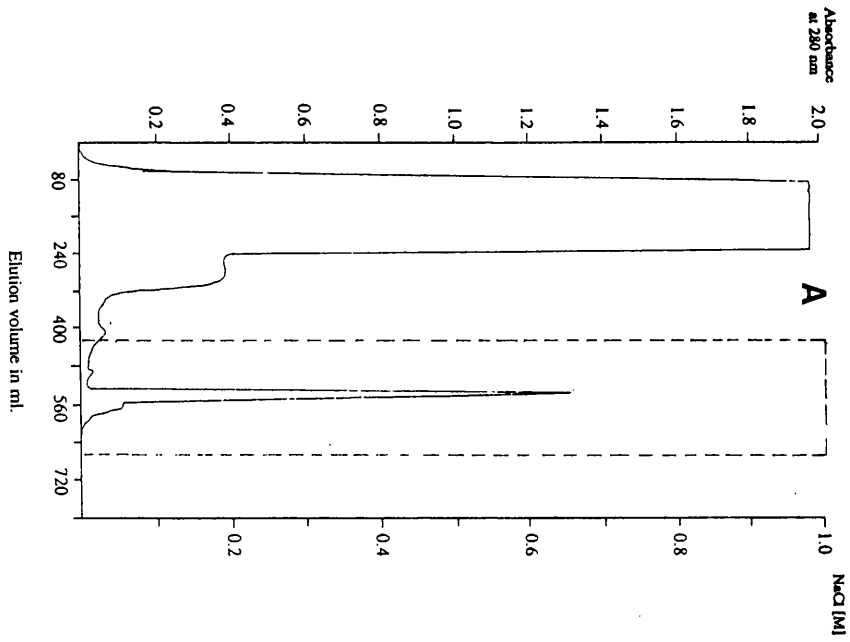
**Figure 4.16:** Purification of the mutant RNase E111Q (arrow).

The procedure for purifying this mutant has been carried out in the same way as when purifying recombinant RNase (see legend of figure 3.5). However, the mutant RNase E111Q elutes from the mono-S column at 210 mM (later than recombinant RNase) due to the fact that an amino acid with a negative charge (Glu-1110 has been removed and replaced by an amino acid with no charge (Gln).

**A.-** Isocratic elution for isolation of all the positively charged proteins from the periplasm of cultures of *Escherichia coli* harbouring pQR 173.

**B.-** Elution profile for the isolation of the mutant RNase E111Q (arrow).

Figure 4.16



The technique of RCPCR for site-directed mutagenesis is advantageous when compared to conventional techniques. Normally M13 vectors are utilized to incorporate altered DNA sequences. To achieve this, single stranded M13 DNA has to be isolated, followed by hybridization to the oligonucleotide containing the altered sequence coding for a specific amino acid change. Finally extension with Klenow is required to produce transformable DNA (double strand). Single stranded DNA is then isolated and used as template for sequencing. Once the sequence has been confirmed, the gene of interest has to be obtained from the replicative form and ligated into an expression vector. This procedure involves many manipulations and is time consuming.

The technique of RCPCR involves: synthesis and purification of primers, two PCR reactions, electrophoresis and extraction of the products (this is an optional step, which is used to prevent carry over of parental plasmid). The two double stranded DNA fragments are then combined, denatured and annealed. Finally transformation with the "nicked" or "gapped" recombinant circles is carried out. The plasmid content of the recombinant colonies is isolated and used for double stranded DNA sequencing (if the pUC series is utilized). If the sequence is correct (i.e. the mutation has been incorporated and no spurious mutations is detected), ligation of the mutated gene into an expression vector is performed.

The expression system described in Chapter 3 allows the production of mutant RNases in less than 48 hours with yields sufficient for carrying out the kinetic analysis which will be described in Chapter 5.

The importance of having an expression system that produces exogenous protein in such a short time, and a technique for site-directed mutagenesis that involves a small number of manipulations, is that the assessment of the kinetic behaviour of any mutant towards different dinucleotide substrates can be achieved in 15 days.

## CHAPTER 5: KINETIC CHARACTERIZATION OF RECOMBINANT RNase AND ITS GENETICALLY ENGINEERED MUTANTS

Significant technical problems have been encountered during the kinetic characterization of the commercial and recombinant RNase and genetically engineered mutants presented in this chapter. The dinucleotide substrates used (CpA, CpG, CpC and CpU) are extremely expensive (£47/ mg) and the equipment utilized for the spectrophotometric assay was limited in its ability to monitor the hydrolysis of high concentrations of substrates. For this reason only a complete kinetic characterization of the RNases described previously (see Chapter 4) has been carried out using CpA and cytidine 2':3' cyclic phosphate as substrates. The CpA used in these kinetic determinations was synthesized in this laboratory by Dr. H. J. Eggelte (see Appendix 1). Due to the limited availability of the substrates CpG, CpC and CpU it has only proved possible to determine initial velocities for each enzyme using one concentration of each of these substrates.

To relate reaction rate to the concentration of free rather than total enzyme, use has been made of the ratio  $k_{cat}/K_m$  (a measure also of the efficiency with which the enzyme turns over substrate). However, determination of  $k_{cat}/K_m$  directly from the progress curve has not been possible since the product of transesterification, the intermediate, cytidine 2':3'- cyclic phosphate is itself a substrate for the enzyme and its hydrolysis product, cytidine 3'- monophosphate can act as an inhibitor (Davis and Allen, 1955) (see Fig. 5.1). It has therefore proved necessary to use other techniques to obtain  $k_{cat}$  and  $K_m$  values for the different RNases described here using CpA and cytidyl 2':3'-cyclic phosphate as substrates.

The mechanism of the hydrolysis of dinucleotides takes place in two steps:

1) Transesterification: A chain cleavage resulting in the formation of cytidine 2':3'-cyclic phosphate and a free 5'-OH group at the other side of the bond cleaved (see Fig. 5.1). This step can be monitored by the decrease in absorbance at 286 nm (Witzel *et al.*, 1962) with time or by the increase in absorbance at 265 nm with time.

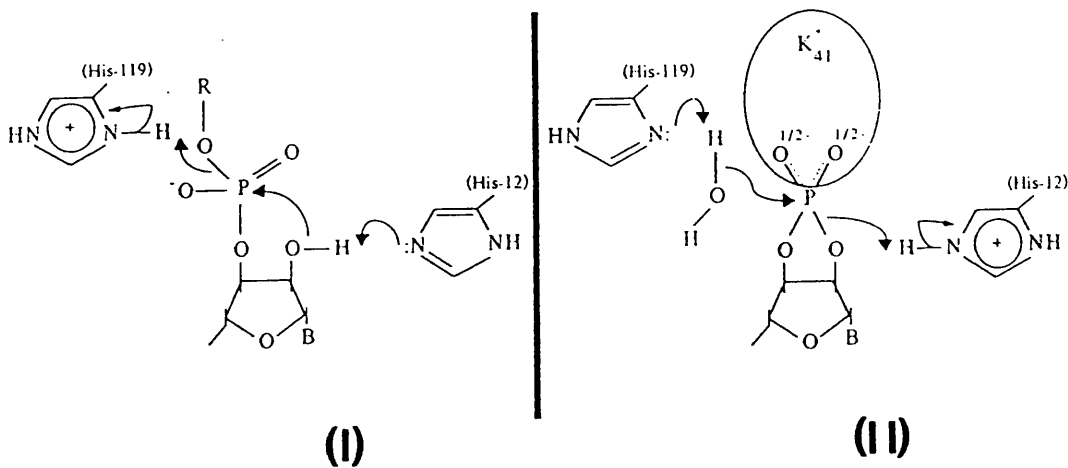
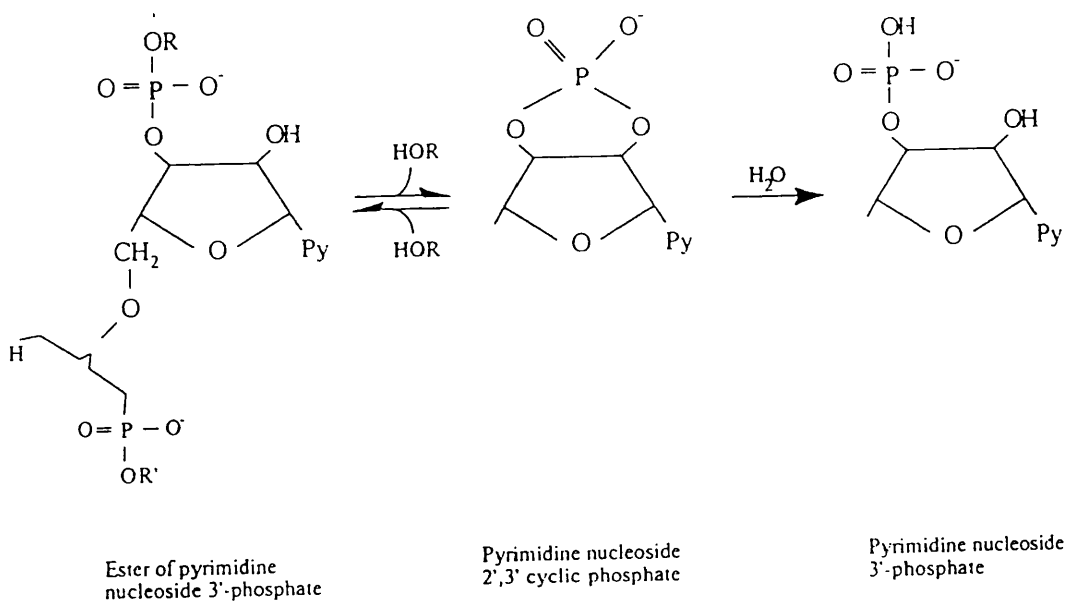
2) Hydrolysis of the cyclic phosphate to yield the 3'-phosphate monoester group (see Fig. 5.1). This step can be monitored by the increase in absorbance at 286 nm with time (Crook *et al.*, 1962).



**Figure 5.1:** Proposed mechanism for the hydrolysis of dinucleotide substrates by RNase.

His-12 acts as a general base and His-119 as a general acid in the transesterification or step (I) but their roles are reversed when cytidine 2':3'-cyclic phosphate is hydrolyzed to cytidine 3'-phosphate (step II) (Findlay *et al.*, 1962)

Réaction catalyzed by Ribonuclease



### **5.1 Spectra of cytidine 2':3'-cyclic phosphate and cytidine 3'-phosphate.**

A difference spectrum of cytidine 2':3'-cyclic phosphate and cytidine 3'-phosphate was produced as described in section 2.26.2 based on the previous work of Crook *et al.*, (1960). The spectra obtained are shown in Fig. 5.2. The difference in the spectra show that the hydrolysis of cyclic phosphate can be followed by monitoring an increase in absorbance at 286 nm with time and that there is an isobestic point at 265 nm (see Fig. 5.2). The fact that the substrate and the product have the same absorbance at 265 nm indicates that transesterification could be monitored independently of the second step of hydrolysis at 265 nm. In order to ascertain whether this assumption is correct, a series of difference spectra were obtained for the four dinucleotides (CpA, CpG, CpC and CpU).

### **5.2 Spectra of CpA, CpG, CpC and CpU and their RNase hydrolysis products.**

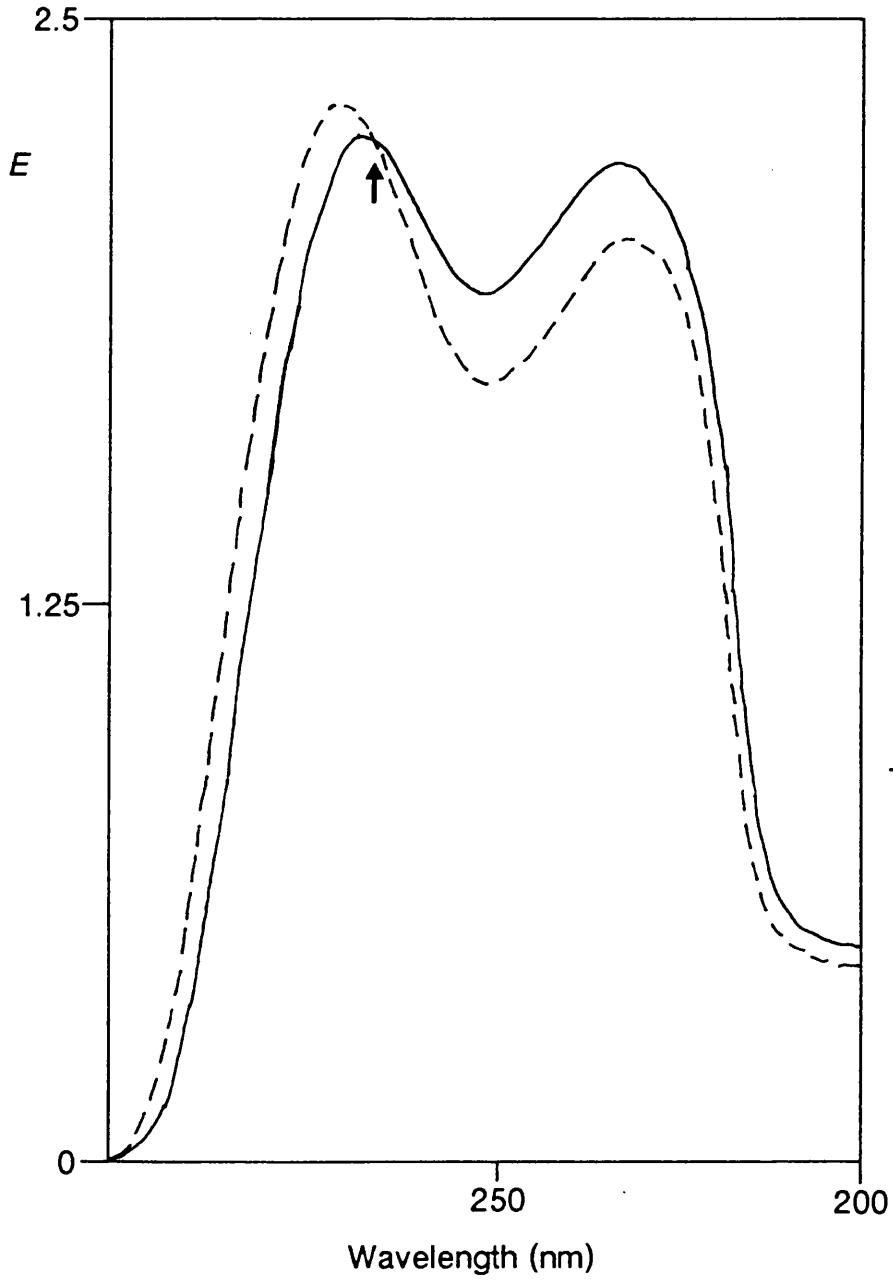
A series of assays containing dinucleotide substrates were set up as described in section 2.26.3., and a difference spectrum was obtained by taking a spectrum before and after a 10 min. hydrolysis (see Fig. 5.3). It is clear from these spectra that the hydrolysis of dinucleotides can be monitored by an increase in absorbance at 265 nm with time. At this wavelength, the formation of the cyclic phosphate is measured and subsequent hydrolysis to cytidine 3'- monophosphate is not detected since this product has the same absorbance as the cyclic intermediate (see Fig. 5.1) (see Fig. 5.2, "isobestic point" (↓)).

**Figure 5.2:** Total hydrolysis of cytidine-2':3'-cyclic phosphate.

The hydrolysis of cytidine-2':3'-cyclic phosphate by RNase was measured using the method of Crook *et al.*, (1960). The assay was carried out in a volume of 1 ml in a cuvette of 1 cm path length (Hellma) and contained 0.3 mM cytidine-2':3'-cyclic phosphate in 0.1 M Tris acetate pH 6.5 ( $I=0.1$ ). The reaction was initiated by addition of RNase to a final concentration of 2.9  $\mu$ M.

A spectrum of cytidine-2':3'-cyclic phosphate was obtained over a wavelength range of 190-300 nm, against a blank containing buffer alone. Enzyme was then added to the sample cuvette and hydrolysis was allowed to continue at room temperature for 2 hours. The reaction was assumed to be complete at this time and a spectrum of the product cytidine 3'-phosphate was obtained. The continuous line is the spectrum obtained before hydrolysis and the dashed line, the spectrum obtained after hydrolysis. The isobestic point at 265nm is clearly seen ( $\uparrow$ ).

Figure 5.2



**Figure 5.3: Difference spectra of the hydrolysis of dinucleotide substrates.**

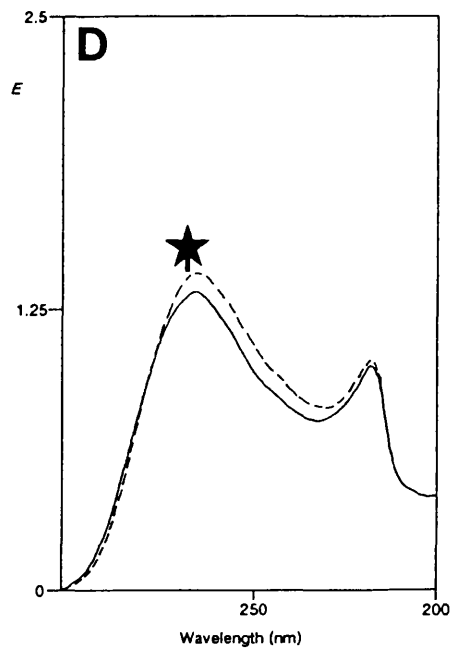
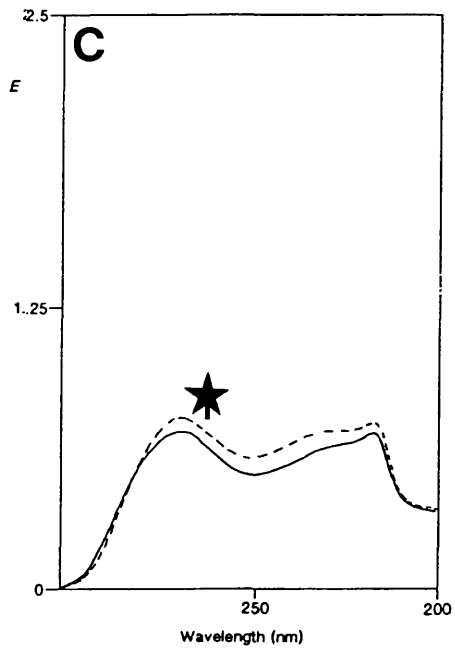
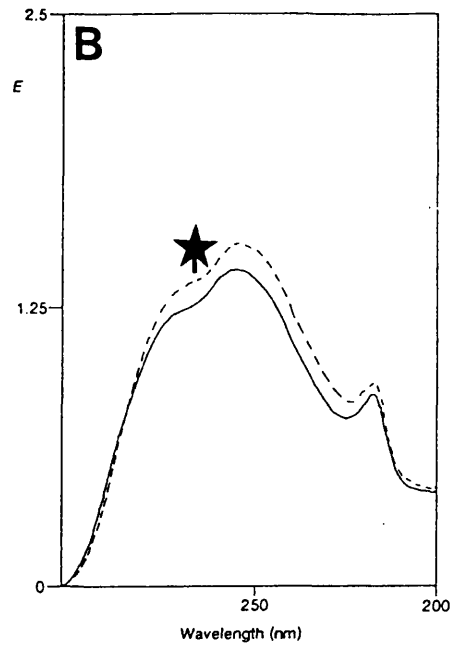
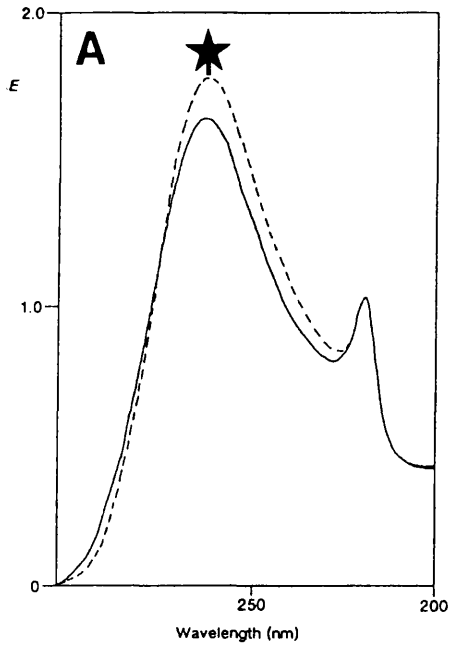
The hydrolysis of CpA, CpG, CpC or CpU by RNase was measured as follows: all assays were carried out in a volume of 1 ml in a cuvette of 1 cm path length (Hellma) and contained 87  $\mu\text{M}$  CpA, 42.5  $\mu\text{M}$  CpG, 45  $\mu\text{M}$  CpC or 45  $\mu\text{M}$  CpU respectively in 0.1 M Tris acetate pH 6.5 ( $I=0.1$ ). Reactions were initiated by the addition of RNase to a final concentration of  $1.4 \times 10^{-7}$  M.

Spectra of CpA ( graph A), CpG (graph B), CpC (graph C) and CpU (graph D) were obtained over a wavelength range of 190-300 nm, against a blank containing buffer alone. Enzyme was then added to the sample cuvette and hydrolysis was allowed to continue for 10 minutes, at which time, a spectrum was obtained of the reaction products.

The continuous line corresponds to the spectrum observed for the different dinucleotide substrates and the dashed line corresponds to the spectrum obtained after 10 minutes of hydrolysis.

★: represents the position of the spectrum at 265 nm, before and after hydrolysis. The clear difference provides the basis for the development of a new series of assays.

Figure 5.3



Thus a novel series of assays can be developed for monitoring the activity of RNase towards the different dinucleotide substrates which has the advantage that only the transesterification step (step I, Fig. 5.1) is monitored. This type of assay at 265 nm overcomes problems associated with the assay of Witzel and Barnard, (1962) where hydrolysis is measured at 286 nm. At this wavelength, the dinucleotide substrate and the products of the hydrolysis have the same absorbance, but the cyclic intermediate has a lower absorbance. Thus assays at 286 nm progress through a decrease in absorbance during transesterification (step I, Fig. 5.1) followed by an increase, during hydrolysis of the cyclic intermediate (step II, Fig. 5.1), to the initial absorbance. This approach is not problematical for the best RNase substrate such as CpA, where the  $k_{\text{cat}}$  for the dinucleotide is high compared to that of the cyclic intermediate (  $3000 \text{ s}^{-1}$  and  $5.5 \text{ s}^{-1}$  respectively) (Witzel and Barnard, 1962). However, for poorer substrates such as CpU where the  $k_{\text{cat}}$  for the dinucleotide approaches that for the hydrolysis of the cyclic intermediate it becomes difficult to measure transesterification rates without an influence from cyclic hydrolysis.

In this chapter, kinetic measurements of commercial, recombinant and mutant RNases using CpA as substrate have been carried out at a wavelength of 286 nm (Witzel et al., 1962). A combination of this wavelength and the use of 0.1 cm path length cuvettes enables hydrolytic assay of CpA to be carried out at concentrations as high as 5 mM. Assays of the other dinucleotide substrates CpG, CpC and CpU were carried out as initial velocity measurements at 265 nm (see Fig. 5.3) and are described in section 5.5.

### **5.3 Kinetic characterization of recombinant RNase and its genetically engineered mutants when using CpA as substrate.**

The double reciprocal plot of Lineweaver and Burk (1934) is commonly used for the determination of the kinetic constants. However, one of the main disadvantages of this plot is that the error of each experimental point has to be known, this is known as the "weight" of each of the experimental points.

In order to overcome this problem, use has been made of the direct linear plot (Eisenthal and Cornish-Bowden, 1974). This type of analysis attempts to introduce a determination that is independent of the distribution of the data. The median value for the data is determined directly from the plot to obtain  $K_m$  and  $V_{max}$  (as  $\Delta OD_{286\text{ nm}}/\text{time}$ ) (see Fig. 5.4). On the X axis is the substrate concentration used and the Y axis is the velocity ( $v$ ) as the change of  $OD_{286\text{ nm}}$  with time. For each observation ( $s$  and  $v$ ), a point is marked on the -X and Y axis and a line is drawn through pairs of each observations extending into the second quadrant. When this is done for all observations, a series of intersections appear in the first quadrant (see Fig. 5.4). A procedure is then followed to find out the best value for  $K_m$  and  $V_{max}$ . Each intersection is considered to provide an estimate of  $K_m$  and  $V_{max}$ , the median value from each series is taken to be the best estimate of  $K_m$  and  $V_{max}$ .

The advantages of using this plot are that it is far less dependent on assumptions about the nature of experimental error and that it uses the median value instead of the mean to find the best estimate of  $K_m$  and  $V_{max}$ . The use of the median value is useful in that the extreme or aberrant values become neutralized.

This type of plot has been used to determine the  $K_m$  of commercial, recombinant and the genetically engineered mutant enzymes for CpA and cytidine 2':3 -cyclic phosphate. A representative example of this type of graph is shown in Fig. 5.4. The different concentrations of substrate (CpA) used are represented on the -X axis and  $v$  is represented on the Y axis. The enzyme used was commercial RNase. The value for the  $K_m$  of commercial RNase for CpA obtained from this plot was 4.2 mM (see Fig. 5.4). The  $K_m$  of the different mutants for CpA and the cyclic intermediate are presented in tables 5.2 and 5.3 respectively.

The assays were carried out as described in section 2.26.4. The highest concentration of substrate that was used was 5 mM. The absorbance of the substrate above this value exceeded the upper limit of the spectrophotometer. Thus the range of substrate



concentration used was from 1 to 5 mM (see Fig. 5.4) or 0.5 to 4 mM in 5 independent kinetic measurements for the recombinant and mutant enzymes. Different concentrations of enzyme have been used due to the variable effect of the mutations on CpA hydrolysis (see Table 5.1).

The direct linear plot is used to obtain  $K_m$  by projecting a line from the median intersection to the X axis (see Fig. 5.4). The catalytic constant  $k_{cat}$  was determined by allowing the reaction to proceed to completion. The absorbance of CpA at 286 nm is the same as the mixture of cytidine 3'-phosphate and adenosine however the absorbance of the mixture of cytidine 2':3'-cyclic phosphate and adenosine is lower. If transesterification (step I, Fig. 5.1) is fast enough compared to hydrolysis (step II, Fig. 5.1), the consequence of this is that the progress curve first decreases, reaches a plateau and then starts increasing very slowly towards the origin as the cytidine 2':3'-cyclic phosphate is hydrolyzed to cytidine 3'-phosphate. By extrapolation to  $t=0$  and taking into account the increase in absorbance at 286 nm due to the hydrolysis of the cyclic intermediate, the total change in absorbance and therefore the extinction coefficient ( $\epsilon$ ) can be obtained. The extinction coefficient thus calculated and the  $V_{max}$  value measured as the change in OD at 286 nm with time obtained from the direct linear plot by projecting a line from the median intersect to the ordinate axis (see Fig. 5.4) is then applied to obtain  $V_{max}$  (as mols of substrate converted per second) using the equation  $OD = \epsilon cl$ . In this equation the OD is the value obtained from the direct linear plot,  $\epsilon$  is the extinction coefficient obtained from the total change in OD that is produced by the complete hydrolysis of a given concentration of substrate, and  $l$  is the path length of the cuvettes used in the assay. The result obtained  $c$  (moles of substrate converted per second or  $V_{max}$ ) is then used to find  $k_{cat}$  by substitution into the equation,  $V_{max} = k_{cat}E_0$ , since  $E_0$  is known. The kinetic parameters obtained from commercial, recombinant and mutant RNase calculated as described above are presented in Table 5.2.

**Figure 5.4:** Direct linear plot of 5 observations (s and v) for commercial RNase

The direct linear plot treats s and v as constants and  $K_m$  and V as variables.

v is the initial velocity as  $\Delta OD_{286}/\text{min}$ . and the concentration of CpA [-S] (mM). Each line represents one observation of the rate of the reaction and is drawn with intercepts -S on the abscissa and v on the ordinate. Each intersection (•) gives an estimate of  $K_m$  and V. The value of  $K_m$  was obtained from the median intersect. The value of  $k_{\text{cat}}$  is obtained as explained in section 5.3.

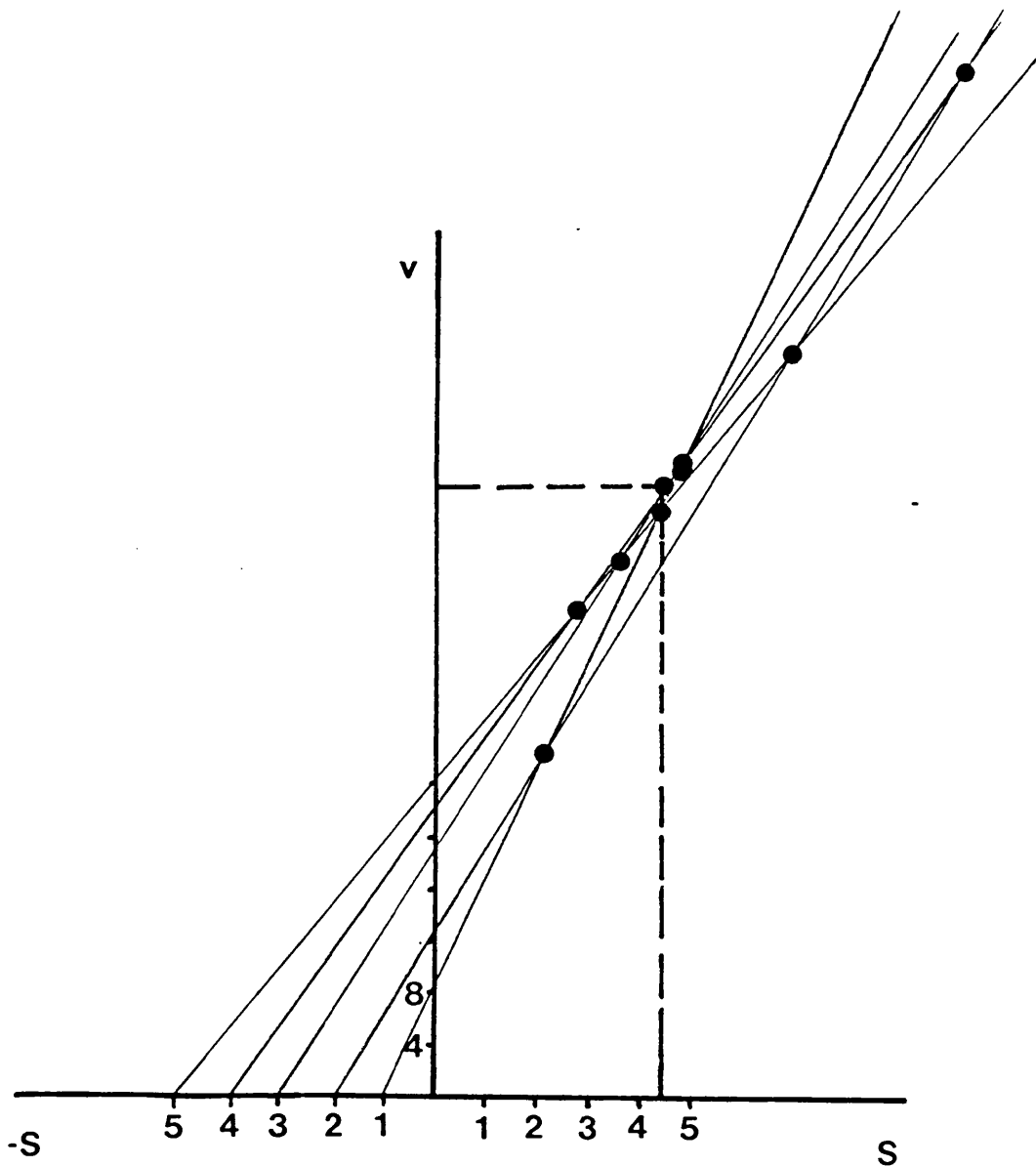


Table 5.1: Final concentration of each different enzyme used in the assays (see section 5.3)

	Final concentration of enzyme (nM) used in assay
Commercial RNase	0.88
Recombinant RNase	0.88
K41R RNase	88
Q69A RNase	0.43
N71A RNase	44
Q69A:N71A RNase	44
E111Q RNase	0.88

Table 5.2: Kinetic values for recombinant RNase and its genetically engineered mutants when using CpA as substrate.

	$k_{cat}$ ( $s^{-1}$ )	$K_m$ (mM)	$k_{cat}/K_m$ ( $mM^{-1}s^{-1}$ )
Commercial RNase	11200	4.2	2700
Recombinant RNase	11600	4.3	2700
K41R RNase	17	3.1	5.5
Q69A RNase	9500	8	1200
N71A RNase	500	7.5	67
Q69A:N71A RNase	700	11.5	61
E111Q RNase	9100	3.65	2500

From the data shown in Table 5.2 we can conclude that the procedures for the expression and purification of recombinant RNase in *Escherichia coli* do not affect the kinetic characteristics of the enzyme since they are equivalent to those of the commercial enzyme (see table 5.2). Thus, we can presume that different activities of mutant enzymes for the same substrate when compared to commercial or recombinant RNase, are due to the engineered mutation and not to the procedures used for the production and isolation of the mutant enzyme. The effects of the engineered mutations on the hydrolysis of CpA are listed below:

1) The effect of replacing Lys-41 by Arg is a large decrease in  $k_{cat}$  while  $K_m$  hardly changes when CpA is used as substrate. This is consistent with the results of Trautwein *et al.*, (1991) who also found a large reduction in  $k_{cat}$  for the dinucleotide substrate uridine-3':5'-adenosine and a large decrease in the hydrolysis of the cyclic. This result is also consistent with the postulated role of the residue Lys-41, in the stabilization of one of the intermediates of the reaction pathway (Rabin, 1990) (see Fig. 5.1).

2) A minor effect is observed when Gln-69 is replaced by Ala. The  $K_m$  increases by a factor of 2 whilst the  $k_{cat}$  is slightly reduced, giving a ratio ( $k_{cat}/K_m$ ) that is half of that observed for the commercial and recombinant enzyme.

3) When Asn-71 is replaced by Ala a 20-fold decrease in  $k_{cat}$  is observed while the  $K_m$  is raised by a factor of 2.

4) The RNase enzyme with the double mutation Q69A:N71A has a similar  $k_{cat}$  to that of the RNase N71A mutant for the hydrolysis of CpA, but the  $K_m$  is raised by a factor nearing 3 with respect to commercial or recombinant RNase.

5) No significant changes are observed when Glu-111 is replaced by Gln. The mutated enzyme has a similar  $K_m$  and a only a small reduction in  $k_{cat}$  is noticed when CpA is used as a substrate. The ratio  $k_{cat}/K_m$  hardly changes in comparison to that for the commercial or recombinant enzyme.

The fact that the equipment utilized did not permit the use of concentrations of substrate near (for some mutants) or above the  $K_m$  could slightly modify these values. However, very clear facts can be inferred from these results. It is clear that Asn-71 is of critical importance for the hydrolysis of CpA, also Gln-69 can be considered as non-critical, as evidenced by the fact that the mutation of both amino acids to alanine (double mutant) produces an enzyme which behaves similarly to the mutant RNase N71A (see  $k_{cat}/K_m$  ratio in table 5.2). The enzyme produced when Glu-111 is changed to Gln, exhibits no significant difference in its ability to hydrolyze CpA. However a further substitution of Glu-111 for Ala is required to assess the importance, if any, of this amino acid in the hydrolysis of CpA.

Commercial, recombinant and engineered mutants of RNase have been used in assays monitoring the hydrolysis of CpA at 265 nm. These have been carried out as described in section 2.26.6, and the progress curves are represented jointly with the progress curves obtained for the other dinucleotide substrates in Fig. 5.7 and 5.8. It is clear that the trends or effects that the several mutants have on the hydrolysis of CpA are equivalent to those described in this section.

X-ray crystallographic studies using substrate analogues or inhibitors for RNase have been carried out in order to identify which amino acids interact with the leaving group. Wodak *et al.*, (1977) made a crystalline complex of RNase-S (like RNase-A but with no peptide bond between amino acids 20 and 21) and cytidine 2':5'-adenosine by diffusing the inhibitor into pre-grown crystals. A combination of X-ray data obtained at 2 Å resolution and computer graphic modelling were used to elucidate the interactions between the enzyme and the leaving group. They concluded that Asn-71 and Gln-69 hydrogen bond to the N(6) of the adenine ring and that Glu-111 interacts with the N(1) of the adenine ring.

These conclusions are in conflict with the results presented here. When Glu-111 is changed to Gln, a mutant enzyme is produced which hydrolyzes CpA as efficiently as

commercial or recombinant RNase (see  $k_{\text{cat}}/K_m$  ratios in Fig. 5.2). Gln-69 has a very minor effect, its substitution by alanine does not seem to affect to a great extent the hydrolysis of CpA. However, Asn-71 does appear to have a clear role in the binding of the adenine ring, the  $k_{\text{cat}}/K_m$  ratio drops by a factor of 40 when this amino acid is changed to alanine. The interaction of Asn-71 has with the adenine ring could possibly occur through one or more hydrogen bonds. However, there is also the possibility that Asn-71 assists another amino acid in the vicinity to interact with the adenine ring.

Other groups have also postulated similar interactions. Brünger *et al.*, (1985) have used the stochastic boundary molecular method to elucidate what interactions the enzyme has with the adenine ring. This method involves the utilization of a known X-ray structure, the substrate (CpA) is then fitted into it taking into account water and buffer molecules. It was concluded from their findings that Asn-71 and Asn-67 interact with the N(6) of the adenine ring and that the former and Gln-69 also interact with the N(1) of the adenine ring.

Other reported interactions come from the studies carried of Llorens *et al.*, (1989). who have studied the enzyme complexed with a pentanucleotide substrate of known sequence (pApUpApApG) by means of model building and computer graphics. From their findings, it is clear that the adenine ring is placed in the B2 subsite (see Chapter 1). This subsite is formed by Gln-69 opposite the N(6) of adenine, Asn-71 adjacent to N(1) of the adenine ring and Asn-67 and Asp-121 close to the N(7) of the base. Glu-111, Val-108 and the disulfide bridge 65-72 are also near this subsite.

All these groups report similar interactions between the enzyme and the leaving group. However there is no consensus about the position of the adenine ring when interacting with a given amino acid. Thus, for example, the N(1) of the adenine ring could interact with Glu-111 (Wodak *et al.*, 1977), or with Gln-69 (Brünger *et al.*, 1985) or even with Asn-71 (Brünger *et al.*, 1985 and Llorens *et al.*, 1989). The data

presented in this thesis agree with these groups on the importance of Asn-71. The amino acid Gln-69 has been reported by all the groups to have a direct interaction with the base. However, the results reported here indicate the contrary or at least that its role is not very well defined. To elucidate the importance of Glu-111, it will be interesting to substitute it with alanine. However the substitution for Gln does not seem to have an effect on the hydrolysis of CpA. The role of Glu-111 will be discussed in more detail in section 5.5.

The exact nature of the interaction of Asn-71 with (N1) or (N6) of the adenine ring is not known. It is possible that it is not interacting directly but assists in the interaction of another amino acid with the base. However, evidence from other laboratories suggests that a hydrogen bond is produced. Brünger *et al.*, (1985) have laid down strict criteria for the interactions of the amino acids with the adenine ring, these being that the donor-acceptor distance has to be  $< 3.5 \text{ \AA}$  and the donor-hydrogen-acceptor angle is  $< 65^\circ$ .

Much work has been carried out involving the amino acids that form part of the B2 subsite. Vallee *et al.*, (1989) have carried out extensive work with angiogenin. This protein is an RNase-like enzyme although it has very little RNase activity. It appears that the reasons for this relate to changes in the region between residues 65 and 72 and the absence (in angiogenin) of the disulfide bond between amino acids 65 and 72. Regional mutagenesis has generated a covalent angiogenin-RNase hybrid protein, where residues 58-70 of angiogenin are replaced by the corresponding segment of RNase (residues 59-73). This hybrid has a 200-fold enhanced activity towards CpA when compared to normal angiogenin, and its "*in vivo*" angiogenicity is markedly decreased (see Chapter 1) (Harper and Vallee, 1989).

The work of Pavlovsky *et al.*, (1978) shows that this region (65-72) is important for the binding of dinucleotide substrates. A crystal complex of RNase-S with the substrate analogue 2'-deoxy-2'-fluoro uridilyl 3':5'-adenosine (2'-F--dUpA) was

prepared and it was found that a change of the sulphur atom of Cys-65 upon binding of the substrate analogue, creates space for the binding of the adenine ring.

It is important to note that these residues in the region 65-72 are conserved in most RNases. However, none of the residues in the region 65-72 in angiogenin are similar to those of RNase, with the exception of Asn-69 (equivalent to Asn-71 in RNase). This indicates that apart from Asn-71, other aminoacids in the region 65-72 of RNase could be very important for the hydrolysis of dinucleotides since a 200-fold increase is observed upon replacement of this region in angiogenin. Attempts to prove this assumption would involve the substitution of Asn-67 by Ala. All this evidence denotes the importance of this disulfide bridge which joins amino acids 65-72 thereby producing a flexible loop in the structure (Carlisle *et al.*, 1974). This loop would provide enough mobility for the amino acids comprising it to interact with the leaving group of the different dinucleotide substrates.

#### **5.4 Kinetic characterization of recombinant RNase and its genetically engineered mutants when using cytidine 2':3'-cyclic phosphate as substrate.**

The conditions for the assay are described in section 2.26.5. The results presented in section 5.3 indicate that replacement of Asn-71 with Ala has a very clear effect on the hydrolysis of CpA. However, the data presented do not shed light on the interactions that Asn-71 makes with the dinucleotide substrate. There are two possibilities, either by assisting in the binding of the pyrimidine or interacting with the leaving group.

To elucidate the function of Asn-71, it is necessary to observe what effect the mutation has on the hydrolysis of cytidine 2':3'-cyclic phosphate. This was carried out for the rest of the mutants and for the commercial and recombinant enzymes.

The direct linear plot described in section 5.3 has been used for the determination of the  $K_m$  values of each enzyme for the substrate cytidine 2':3'-cyclic phosphate. The catalytic constant was determined as described in section 5.3. The range of substrate



concentrations used was from 2-6 mM in 5 independent kinetic assays. Concentrations of substrate above 6 mM have an absorbance that exceeds the upper resolution limit of the equipment used.

Preliminary assays at 286 nm were carried out for each mutant enzyme using cytidine 2':3' cyclic phosphate as substrate. They were performed in order to ascertain whether the engineered mutation had any effect on the hydrolysis of the cyclic intermediate (step II, Fig. 5.1). The progress curves are shown in Figs. 5.5 and 5.6. It is clear that no significant effect is observed in any of the mutant enzymes on the hydrolysis of cytidine 2':3'-cyclic phosphate. These assays were repeated and the kinetic parameters for each enzyme were determined using the direct linear plot (see table 5.3).

The final concentration of each enzyme used was 0.29  $\mu\text{M}$ . The data indicates that the engineered mutations do not change the efficiency ( $k_{\text{cat}}/K_m$ ) with which the enzyme hydrolyses cytidine 2':3'-cyclic phosphate.

We can conclude therefore that the substitutions produced in RNase involving amino acids Gln-69, Asn-71 and Glu-111 do not affect the rate of hydrolysis of the cyclic intermediate. Due to the fact that concentrations of substrate above 6 mM could not be measured, the  $K_m$  values obtained may be slightly altered.

**Figure 3.5:** Hydrolysis of cytidine 2':3'-cyclic phosphate.

Commercial RNase, recombinant RNase, and the mutant RNases Q69A, N71A and Q69A:N71A (2  $\mu$ g) were used in preliminary assays at 286 nm using cytidine 2':3'-cyclic phosphate as substrate.

The assays were performed as in section 2.26.5.

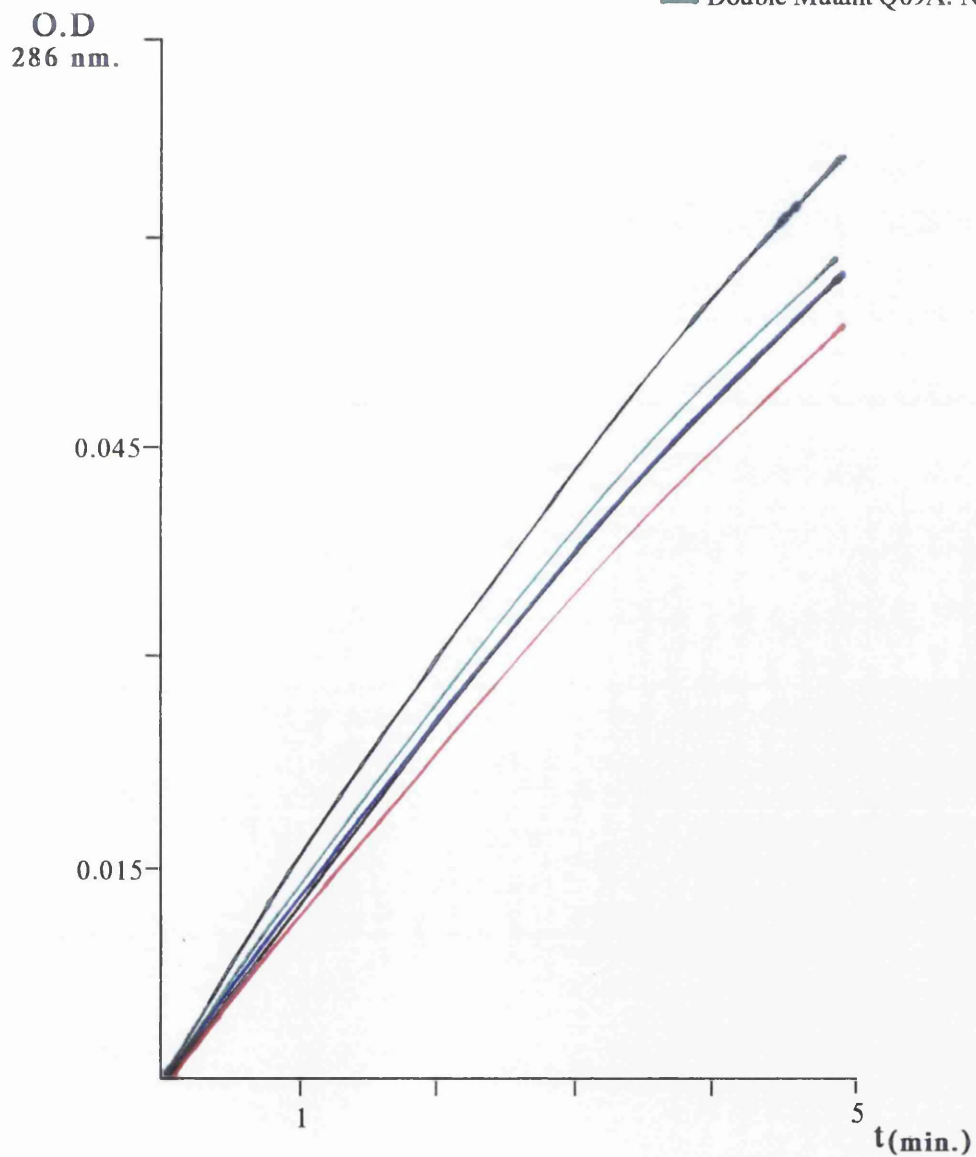
# HYDROLYSIS OF CYTIDINE-2':3'-CYCLIC PHOSPHATE

Concentration of substrate: 1 mM

Enzyme: 2  $\mu$ g

Buffer: 0.1 M Tris Acetate pH 6.5

- Commercial RNase
- Recombinant RNase
- Mutant RNase Q69A
- Mutant RNase N71A
- Double Mutant Q69A: N71A



**Figure 5.6:** Preliminary assays of the hydrolysis of cytidine 2':3'-cyclic phosphate by the mutant RNase E111Q (4  $\mu$ g). The assays were performed as in section 2.26.5.

# HYDROLYSIS OF CYTIDINE-2':3'-CYCLIC PHOSPHATE

Concentration of substrate: 1 mM

Enzyme: 4  $\mu$ g

Buffer: 0.1 M Tris Acetate pH 6.5

--- Commercial RNase

— Mutant RNase E111Q

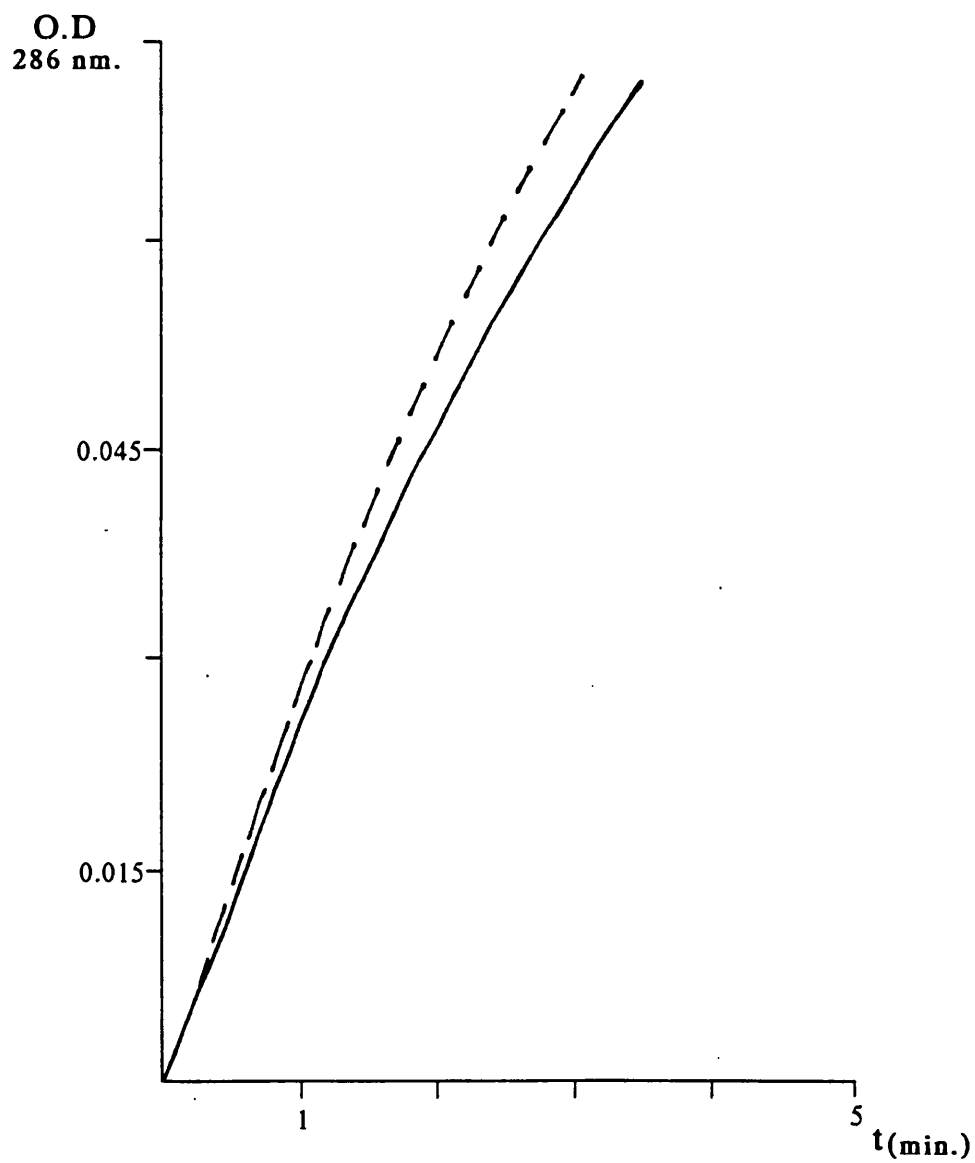


Table 5.3: Kinetic values for recombinant RNase and its genetically engineered mutants for the hydrolysis of cytidine 2':3'-cyclic phosphate.

	$k_{cat}$ ( $s^{-1}$ )	$K_m$ (mM)	$k_{cat}/K_m$ ( $mM^{-1}s^{-1}$ )
Commercial RNase	20	8.5	2.4
Recombinant RNase	28	7	4
Q69A RNase	15	8	1.9
N71A RNase	6	5.25	1.1
Q69A:N71A RNase	5	3.5	1.4
E111Q RNase	20	11	1.8

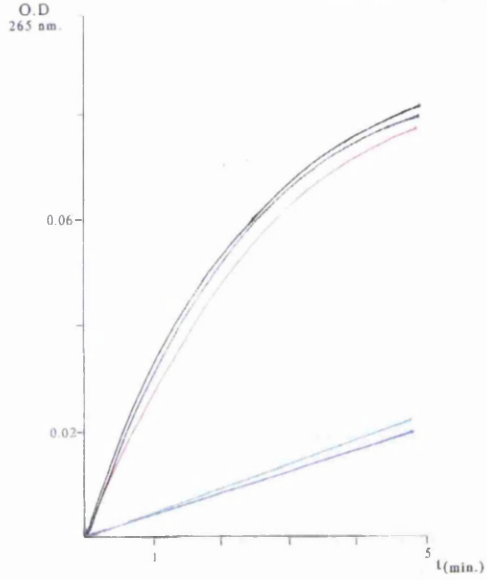
**Figure 5.7: Hydrolysis of the dinucleotide substrates CpA, CpG, CpC and CpU:**

Commercial, recombinant and engineered mutants of RNase (Q69A, N71A and Q69A: N71A) have been used in spectrophotometric assays monitoring the hydrolysis of 0.1 mM CpA, 0.175 mM CpG, 0.182 mM CpC and 0.175 mM CpU at 265 nm. The assays were carried out as described in section 2.26.6. The amount of enzyme used in the assay to hydrolyse a specific dinucleotide substrate was maintained at a constant concentration.

HYDROLYSIS OF CYTIDYLYL-3':5'-ADENOSINE

Concentration of substrate: 0.1 mM  
 Enzyme: 50 ng  
 Buffer: 0.1 M Tris Acetate pH 6.5

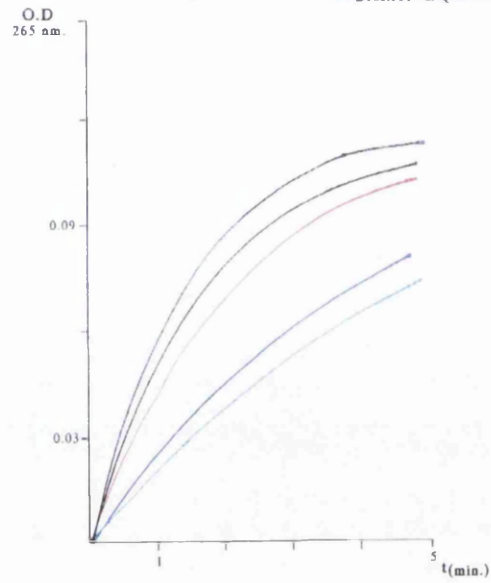
- Commercial RNase
- Recombinant RNase
- Mutant RNase Q69A
- Mutant RNase N71A
- Double Mutant Q69A: N71A



HYDROLYSIS OF CYTIDYLYL-3':5'-GUANOSINE

Concentration of substrate: 0.175 mM  
 Enzyme: 1 µg  
 Buffer: 0.1 M Tris Acetate pH 6.5

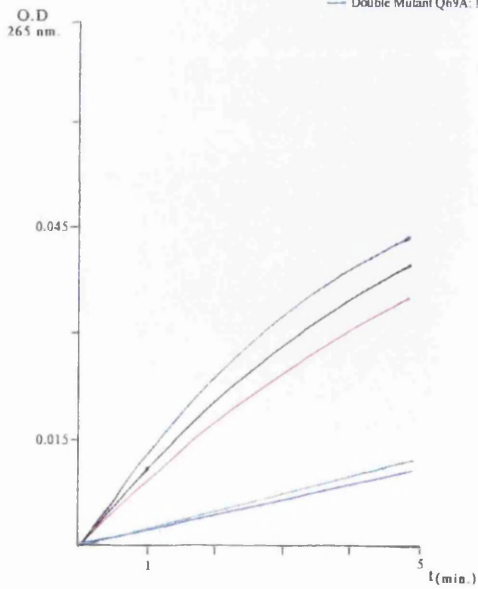
- Commercial RNase
- Recombinant RNase
- Mutant RNase Q69A
- Mutant RNase N71A
- Double Mutant Q69A: N71A



HYDROLYSIS OF CYTIDYLYL-3':5'-CYTOSINE

Concentration of substrate: 0.182 mM  
 Enzyme: 1 µg  
 Buffer: 0.1 M Tris Acetate pH 6.5

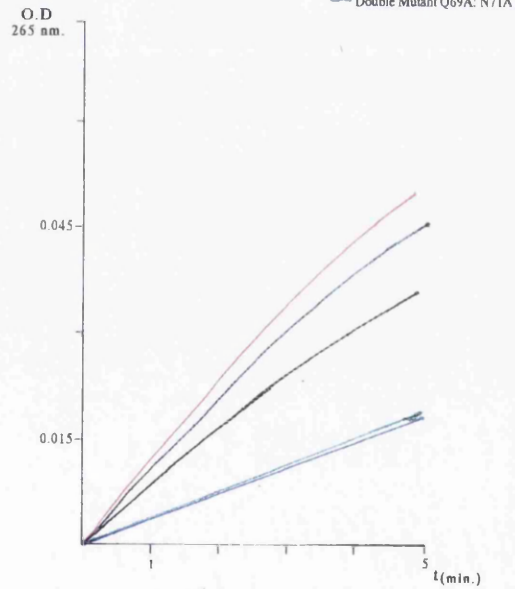
- Commercial RNase
- Recombinant RNase
- Mutant RNase Q69A
- Mutant RNase N71A
- Double Mutant Q69A: N71A



HYDROLYSIS OF CYTIDYLYL-3':5'-URIDINE

Concentration of substrate: 0.175 mM  
 Enzyme: 2 µg  
 Buffer: 0.1 M Tris Acetate pH 6.5

- Commercial RNase
- Recombinant RNase
- Mutant RNase Q69A
- Mutant RNase N71A
- Double Mutant Q69A: N71A





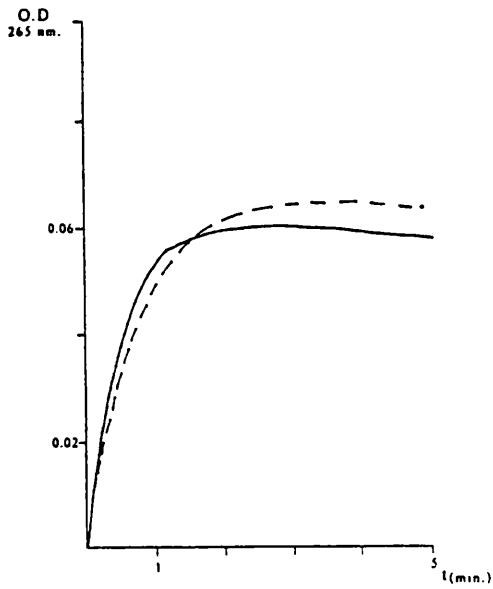
**Figure 5.8:** Hydrolysis of the dinucleotide substrates by the mutant RNase E111Q.

Commercial RNase and the mutant RNase E111Q have been utilized in spectrophotometric assays monitoring the hydrolysis of dinucleotide substrates at 265 nm (see Fig. 5.7 for final concentrations of the substrate used in the assays). The assays were carried out as described in 2.26.6. The concentration of enzyme used in the assay to hydrolyze a specific dinucleotide substrate was maintained at a constant concentration.

HYDROLYSIS OF CYTIDYLYL-3':5'-ADENOSINE

Concentration of Substrate : 0.1 mM  
 Enzyme: 50 ng  
 Buffer: 0.1 M Tris Acetate pH 6.5

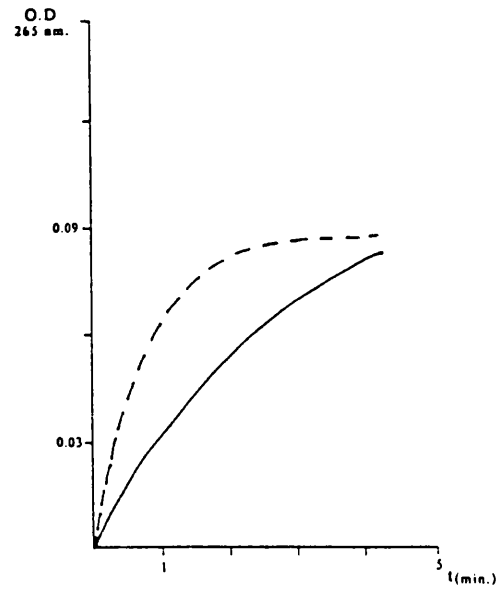
--- Commercial RNase  
 — Mutant RNase E111Q



HYDROLYSIS OF CYTIDYLYL-3':5'-GUANOSINE

Concentration of Substrate : 0.175 mM  
 Enzyme: 1 µg  
 Buffer: 0.1 M Tris Acetate pH 6.5

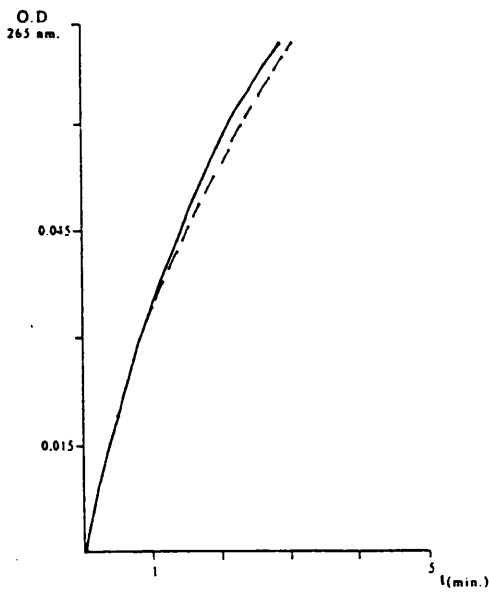
--- Commercial RNase  
 — Mutant RNase E111Q



HYDROLYSIS OF CYTIDYLYL-3':5'-CYTOSINE

Concentration of Substrate : 0.182 mM  
 Enzyme: 1 µg  
 Buffer: 0.1 M Tris Acetate pH 6.5

--- Commercial RNase  
 — Mutant RNase E111Q



HYDROLYSIS OF CYTIDYLYL-3':5'-URIDINE

Concentration of Substrate : 0.175 mM  
 Enzyme: 2 µg  
 Buffer: 0.1 M Tris Acetate pH 6.5

--- Commercial RNase  
 — Mutant RNase E111Q

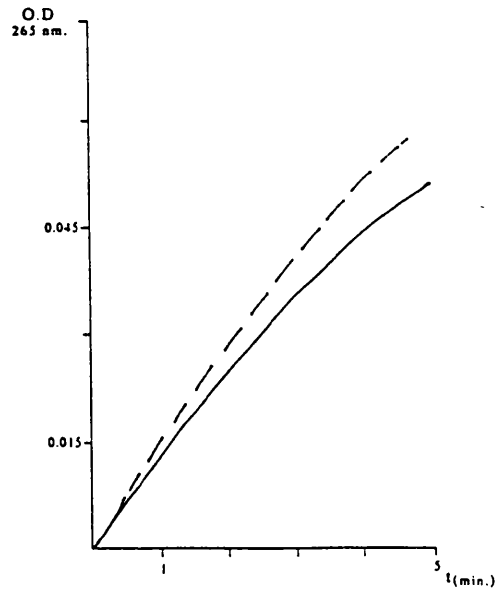


Table 5.4: Final concentration of the enzymes used in the hydrolysis of 0.2 mM CpG, CpC and CpU.

	CpG (0.2 mM)	CpC (0.2 mM)	CpU (0.2 mM)
Commercial RNase	14.6 nM	29 nM	0.29 $\mu$ M
Recombinant RNase	14.6 nM	29 nM	0.29 $\mu$ M
K41R RNase	16.4 $\mu$ M	3.27 $\mu$ M	11.4 mM
Q69A RNase	14.6 nM	29 nM	0.29 $\mu$ M
N71A RNase	58.5 nM	0.41 $\mu$ M	3.14 $\mu$ M
Q69A:N71A RNase	58.5 nM	0.29 $\mu$ M	2.16 $\mu$ M
E111Q RNase	58 nM	29 nM	0.29 $\mu$ M

### **5.5 Preliminary kinetic studies for commercial and recombinant RNase and its engineered mutants when using CpG, CpC and CpU as dinucleotide substrates.**

The conditions of the assay are described in section 2.26.6. Due to the high cost of dinucleotides (£47/mg), the amount of work that could be done was very limited. Preliminary assays of the engineered mutants of RNase were carried out to observe the degree of influence that a specific substitution has on the rate of hydrolysis of the dinucleotide substrates. The progress curves are presented in Figs. 5.7 and 5.8. Once the trends of the mutant enzymes were observed, these assays were repeated using different concentrations of mutant enzymes (see table 5.4). From these latter assays, the rates of hydrolysis were obtained and only initial velocity values expressed as moles of product/sec/mol of enzyme are presented in this thesis (see table 5.5).

From the data presented in table 5.5 we can summarise the following:

- 1) Commercial and recombinant RNase behave in a similar manner in the hydrolysis of the different dinucleotide substrates.
- 2) The substitution of Lys-41 for Arg has a marked effect on the rate of hydrolysis of the dinucleotides.
- 3) The mutation Q69A causes a small decrease in the rate of hydrolysis of CpG and CpC but not that of CpU.
- 4) The mutation N71A and the double mutation Q69A:N71A causes a decrease in the rate of hydrolysis of CpG, CpC and CpU.
- 5) However, the most relevant value is that obtained for the mutation E111Q on the rate of hydrolysis of CpG where a significant decrease is observed. This mutant enzyme does not effect the rate of hydrolysis of CpC and CpU when compared to commercial or recombinant RNase. As there is no effect on the hydrolysis of cytidine 2':3'-cyclic phosphate (see table 5.3) or on the hydrolysis of CpA (see table 5.2), we can conclude that this amino acid interacts specifically with the guanine.

Table 5.5: Initial velocity values for recombinant RNase and its genetically engineered mutants on the hydrolysis of CpG, CpC and CpU. Data obtained from a single experiment. The units for the initial velocity measurements are mols of product produced/sec/mols of enzyme. These units have been chosen due to the fact that different amounts of enzyme have been utilized to obtain a visible rate assay. Therefore, the values are related to enzyme concentration. The substrate concentration when using CpG, CpC and CpU have remained constant (0.2 mM) for each enzyme.

	Vo (0.2 mM CpG)	Vo (0.2mM CpC)	Vo (0.2 mM CpU)
Commercial RNase	60	15	2.7
Recombinant RNase	60	20	2.5
K41R RNase	0.2	0.1	0.01
Q69A RNase	35	11	4.4
N71A RNase	8	1.5	0.5
Q69A:N71A RNase	8	1.4	0.5
E111Q RNase	7.5	17	2.9

It is not possible to ascertain from these data, however, if the changes in activity observed in the mutants are due to a change in  $K_m$  and/or  $k_{cat}$ .

This set of results again show that Asn-71 is a key amino acid involved in the hydrolysis of dinucleotide substrates, and Gln-69 is of minor importance. However, a striking result is the effect that the mutation of Glu-111 to glutamine has on the enzyme when using CpG as substrate. The E111Q RNase mutation decreases the rate of hydrolysis of CpG, but not of the other dinucleotide substrates (including CpA, see section 5.3). Because the hydrolysis of the cyclic intermediate is maintained unchanged, we can conclude that Glu-111 is an amino acid that is involved in interactions with the guanine base.

Some light is beginning to be shed on the specificity that the enzyme shows in the binding of dinucleotide substrates. The substitution of Asn-71 by alanine produces a mutant enzyme which does not hydrolyze any of the dinucleotides at the same rate as commercial or recombinant RNase. The substitution of Glu-111 by glutamine produces an enzyme that hydrolyzes all the dinucleotides, except CpG, as efficiently as commercial or recombinant RNase.

Perry, (1987), working with the substrate analogue cytidine-2':5'-guanosine (C-2':5'-G), made a crystal complex by diffusing the substrate into pre-grown crystals of RNase. When studied at 2 Å resolution, the indications were that the guanine ring interacts with Glu-111 (see chapter 3). It was postulated that the interaction would be of an ionic nature. That is, the N(1) of the guanine ring would be protonated upon binding of the dinucleotide substrate and consequently interact with the negative charge of Glu-111. However, conflicting evidence was reported by Aguilar *et al.*, (1991). Working at a resolution of 1.5 Å, this group observed that the guanine ring binds the enzyme in a retrobinding form, suggesting that "...the preliminary analysis was affected both by the limited resolution of the original electron density maps (2 Å) and by preconceptions as to how the inhibitor binding would occur - based on the

classical model...". However the data presented in this thesis supports the conclusions of Perry, (1987). A full assessment, however, needs to be carried out by changing Glu-111 to alanine, thus deleting any possible interaction with the leaving group.

In this chapter, the kinetic behaviour of the recombinant and mutant enzymes has been presented and compared with the work of other authors. It is clear that conclusive data cannot be obtained from X-ray crystallographic or computer modelling studies. The biological evidence presented in this thesis in which genetically engineered mutants of RNase are produced (Chapter 4) using an original expression system in *E.coli* (Chapter 3), and then kinetically characterized by using several dinucleotide substrates, indicates that Asn-71 and Glu-111 are key residues for the hydrolysis of dinucleotide substrates.

However, it is not possible to state with absolute confidence whether these amino acids interact directly with the leaving group (let alone at which position) or whether they act by aiding other amino acids in the establishment of productive interactions.

X-ray crystallography and site-directed mutagenesis are important tools which enable us to get a deeper insight on what happens when the enzyme interacts with its substrate. But, to go further than that from the data presented in this thesis and the data available in the literature would be speculative. Therefore, we can say that Asn-71 is heavily involved in the hydrolysis of the four dinucleotides, Glu-111 is involved in the hydrolysis of CpG (a change to Ala is necessary for a full assessment), and Glu-69 has an undefined role. We may conclude also that these amino acids do not have an overall effect (ratio  $k_{cat}/K_m$ ) on the hydrolysis of cytidine 2':3'-cyclic phosphate.

## CHAPTER 6: DISCUSSION

A large amount of information is available concerning bovine pancreatic RNase (see Chapter 1). However, the diverse specificity that the enzyme shows towards a range of dinucleotide substrates cannot be explained from the available literature. The work presented in this thesis attempts to shed light on the specificity that RNase shows towards dinucleotide substrates.

The production of recombinant RNase and its genetically engineered mutants using the recombinant circle polymerase chain reaction (RCPCR) technique, has allowed the kinetic characterization of these enzymes using dinucleotide substrates. This has allowed the determination of the involvement of some enzyme residues in the facilitation of the hydrolysis of dinucleotides.

### **6.1 Production of recombinant bovine pancreatic ribonuclease A in *E. coli*.**

The use of the natural signal sequence of RNase has proved successful in the export of RNase to the periplasmic space of *E. coli*. This has enabled a rapid purification strategy involving an spheroplastic/osmotic shock procedure (C. French *et al.*, unpublished) and cation-exchange chromatography (see Chapter 3) to be developed.

Recombinant RNase produced in *E. coli* has been found to be as active as the commercial or natural enzyme. The directed production of RNase into the oxidative environment of the periplasmic space of *E. coli* has aided in the establishment of disulfide bridges and the correct folding of mature RNase. N-terminal sequencing has also allowed for the determination of the first few amino acids of the recombinant protein. The results presented indicate that the signal sequence is recognized by *E. coli* and correctly cleaved (see Fig. 3.8).



This approach is novel since all the expression systems utilized in producing RNase thus far have been designed for the production of recombinant RNase intracellularly (in *E. coli*) or for secretion to the media using *Bacillus subtilis* (see Chapter 3).

Very recently a synthetic gene coding for bovine pancreatic ribonuclease has been fused to the signal sequence of murine spleen ribonuclease. Expression studies have shown that this signal sequence is also successful in translocating recombinant RNase into the periplasm of *E. coli* (Schein *et al.*, 1992).

The potential of the expression system described in this thesis has not been fully investigated since no optimization studies have been carried out. The yields of recombinant RNase produced using the strategy developed in this work are in the order of 100  $\mu\text{g}$  per litre of culture. Since RNase is a highly efficient catalyst, these yields are sufficient for carrying out kinetic measurements using dinucleotide substrates. Thus, we can conclude that the system is adequate for this purpose.

Most of the recombinant RNase produced (83%) is found in the periplasmic space of *E. coli* (see Chapter 3), the rest is passively released to the culture medium during growth of the bacterial culture. This is in accordance with the system described by Schein *et al.*, (1992), where it was observed that between 10 and 50% of the recombinant RNase produced is passively secreted to the culture medium. The reasons for this leakage are not understood, however evidence presented in this work indicates that lysozyme treatment is not necessary for the release of mutants of RNase from the periplasm (see Chapter 4). This suggests that RNase is sufficiently small to pass through a partially permeabilized membrane. This leakage could also be found after long periods of bacterial growth, possibly accounting for the release of RNase to the culture medium. This strategy combined with the purification protocol described in Chapter 3 has enabled the purification to homogeneity of a fully active recombinant RNase. This is achieved in 48 hours from the inoculation of the medium.

The purification strategy involves the use of FPLC-compatible columns which have high resolving power.

It is important that recombinant RNase exhibits an activity which is equal to that of the commercial or natural enzyme. Thus, changes in activity brought about by engineered mutations can definitely be ascribed to that mutation and not to events occurring during manipulation of the clones.

## **6.2 Utilization of the RCPCR technique for site-directed mutagenesis.**

Many techniques are available for the introduction of altered coding sequences into a specific gene. The technique chosen to carry out specific mutations in this work was that of RCPCR.

RCPCR is a technique which utilizes PCR incubations to introduce altered coding sequences using primers which contain the desired mutation. The advantage of using RCPCR is that it only involves two separate PCR incubations, isolation and combination of the PCR products, and denaturation and annealing to form circles which are ready to transform into competent cells. The recombinant colonies harbouring the plasmid of interest, which contains the altered DNA coding sequence, are obtained in 2 days (see Chapter 4). Isolation of plasmid DNA from recombinant colonies is carried out to obtain template which is then used for double stranded DNA sequencing (see Chapter 4).

Other site-directed mutagenesis techniques such as the use of M13-derived vectors (Zoller and Smith, 1982) or the overlapping extension PCR-derived technique (Ho *et al.*, 1989), are time consuming and involve many manipulations. The use of RCPCR eliminates many of these steps (see Chapter 1).

A disadvantage which is inherent in the use of PCR is spurious mis-incorporation. This is greatly alleviated by the use of thermostable enzymes with proof-reading activity.

### **6.3 Identification of amino acids which are involved in the hydrolysis of dinucleotides.**

It is clear that His-12, His-119 and Lys-41 are involved in the mechanism of action of RNase (see Chapter 1). From data available in the literature, the amino acids Asn-67, Gln-69, Asn-71 and Glu-111 are postulated to be involved in the binding of the leaving group at the B2 subsite (see Chapter 1). Site-directed mutagenesis studies involving amino acids at positions 69, 71 and 111 have been carried out in this thesis. The biological evidence produced from the kinetic behaviour that RNase mutants exhibit on the hydrolysis of dinucleotide substrates, is that Asn-71 and Glu-111 are important in the establishment of productive interactions.

The mutation of the amino acid Asn-71 to alanine produces a mutant enzyme that has a lesser activity towards all four dinucleotide substrates (CpA, CpG, CpC and CpU) (see Chapter 5). The mutation of residue Glu-111 to Gln produces an enzyme that hydrolyzes CpG at a lower rate than the recombinant or commercial enzyme. However, the rates of hydrolysis of CpA, CpC and CpU by the mutant RNase E111Q remain unchanged when compared to the activities shown by the commercial or natural enzyme (see Chapter 5). Thus a specific interaction seems to take place between Glu-111 and the guanine base, whether this is a direct or indirect interaction is difficult to ascertain. A further mutation to alanine is required for a full assessment of the role that Glu-111 plays. However, this amino acid, as evidenced by X-ray crystallography, is exposed to the solvent, thus its replacement with a hydrophobic residue such as alanine may have an effect on the folding of the protein. Finally, the mutation of the amino acid Gln-69 to Ala produces an enzyme that hydrolyzes dinucleotide substrates similarly to commercial or recombinant RNase. The slight decrease in activity towards CpA, CpG and CpC is not energetically significant (see

Chapter 5). This evidence is supported by results obtained with the RNase mutant containing alanine residues at positions 69 and 71. The behaviour of this double mutant on the hydrolysis of dinucleotide substrates is equivalent to that of the mutant RNase N71A (see Chapter 5).

The mutation of amino acid Lys-41 to Arg produces an enzyme which has the same  $K_m$  for the dinucleotide substrate CpA when compared to the commercial or recombinant enzyme but the  $k_{cat}$  is largely decreased (see Chapter 5). Thus, it seems that Lys-41 is not involved in the primary binding of the substrate but rather in the catalytic mechanism (Rabin, 1990). This probably occurs by an interaction with the reaction intermediates that are produced after binding of the substrate.

It is clear that the role of amino acid Asn-71 is to facilitate productive interactions involved in the highly efficient hydrolysis of the four dinucleotide substrates by RNase. The amino acid Glu-111 specifically facilitates the hydrolysis of CpG (as evidenced by the mutant RNase E111Q) thus contributing to the specificity that the enzyme shows for purines at the B2 subsite (see Chapter 1). The role of the amino acid Gln-69 is not clear, since the mutant RNase Q69A behaves rather similarly to the commercial or recombinant enzyme.

We can also conclude that the mutations involving the amino acids Gln-69, Asn-71 and Glu-111 produce RNase derivatives that do not affect the hydrolysis of the cyclic intermediate.

Other amino acids whose role in the facilitation of the hydrolysis of dinucleotide substrates remains to be investigated are Lys-66 and Gln-67. The large increase in RNase activity observed in derivatives of angiogenin which have undergone regional mutagenesis involving the introduction of the region 65-72 of RNase in angiogenin indicates that other amino acids apart from Asn-71 are involved in the establishment of productive interactions with dinucleotide substrates.

Further work will involve mutagenesis of the amino acid Glu-111 to alanine. This will remove any possible interaction of a hydrophilic nature. However, a possible problem in protein folding may be encountered. Other mutations will involve the substitution of amino acids Lys-66 and Gln-67 by alanines.

The work presented in this thesis has utilized simple techniques to produce a system whereby recombinant eukaryotic RNase and its genetically engineered mutants are produced in a prokaryotic host. Recombinant RNase and its mutants thereof are then kinetically characterized using dinucleotide substrates in novel spectrophotometric assays (see Chapter 5). This combination of techniques has also allowed the kinetic assessment of each RNase mutant to be carried out in less than a month.

Site-directed mutagenesis and X-ray crystallography are powerful tools which in combination can be used for the better understanding of structure-function relationships in a given protein. The biological evidence presented in this thesis involving the role of the RNase residues Asn-71 and Glu-111, in creating positive interactions for the successful hydrolysis of dinucleotide substrates is partially in accordance with the literature available. However, the role assigned to Gln-69 in previous reports is in conflict with the biological evidence presented in this thesis.

A question remains to be asked. Is it possible to determine the nature of the specific interactions that the enzyme establishes with its substrate using X-ray crystallography and site-directed mutagenesis studies?

X-ray crystallography uses non-substrates to obtain crystallized complexes representative of enzyme and substrate, therefore it can not mimic the exact interactions that the enzyme is establishing with a real substrate. Site-directed mutagenesis may identify amino acids which are relevant to the activity of the enzyme, however it can not determine what type of interaction is being affected.

Therefore, the actual instruments that we have for determining specific interactions in productive enzyme-substrate complexes are insufficient. However, a combination of such techniques represent a step forward in the better understanding of the relationship between the structure and function of a given enzyme.

## APPENDIX 1: SYNTHESIS OF CpA.

The synthesis of CpA was carried out by Dr. H. Eggelte. The method used was as follows: 4-N, 2'-O, 5'-O-triacetylcytidine (Norman and Reese, 1985) was converted to the N-diisopropylmethylphosphoramidite and coupled with 6-N-benzoyl, 2',3'-O-methoxyethylidene adenosine (Fromageot *et al.*, 1967), followed by oxidation with t-BuOOH. After hydrolysis of the orthoester with 50% acetic acid to the 3'-acetate, deprotection of the dinucleotide was achieved with 1) boiling t-butylamine and 2) concentrated NH<sub>3</sub>. The CpA obtained was purified on a mono Q HR10/10 anion exchange column (Pharmacia-LKB) with a linear gradient of H<sub>2</sub>O/ 1 M ammonium formate and the appropriate fractions pooled and lyophilized.

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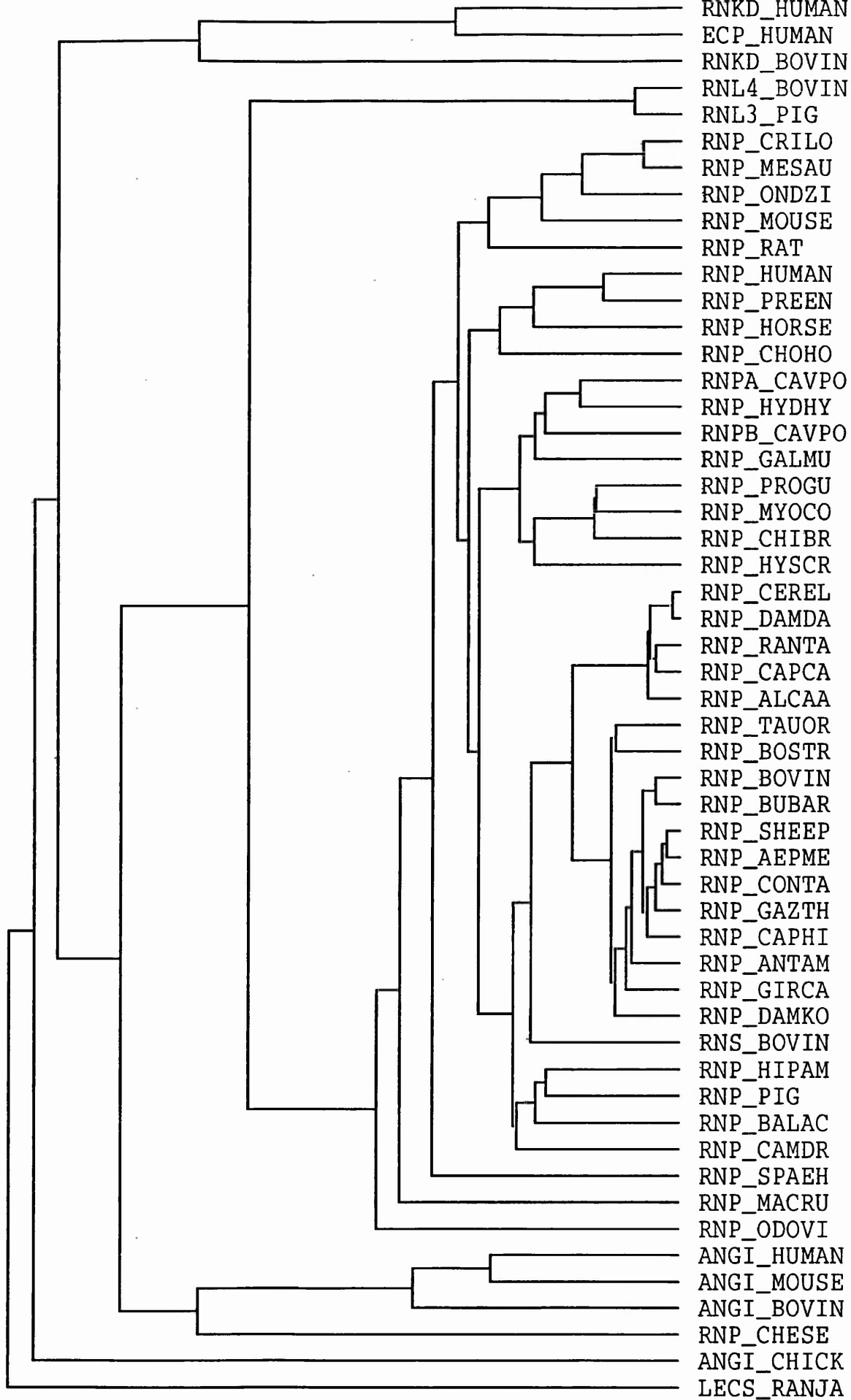
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**APPENDIX II: Evolutionary tree for the pancreatic RNase family and alignment  
of their protein sequences.**

The protein sequences have been obtained from the Swissprot database with the help of Dr. L. Pearl.

## Abbreviations:

**angi\_bovin:** angiogenin from bovine.  
**angi\_chick:** angiogenin precursor from chicken.  
**angi\_human:** angiogenin precursor from human.  
**angi\_mouse:** angiogenin precursor from mouse.  
**ecp\_human:** eosinophil cationic protein precursor from human.  
**lecs\_ranja:** sialic acid-binding lectin from japanese frog.  
**rnkd\_bovin:** ribonuclease K2 from bovine.  
**rnkd\_human:** non-secretory ribonuclease precursor from human.  
**rn13\_pig:** ribonuclease p13 from pig.  
**rn14\_bovin:** ribonuclease b14 from bovine.  
**rnp\_aepme:** ribonuclease pancreatic from impala.  
**rnp\_alcaa:** ribonuclease pancreatic from european moose (elk).  
**rnp\_antam:** ribonuclease pancreatic from pronghorn.  
**rnp\_balac:** ribonuclease pancreatic from minke whale.  
**rnp\_bostr:** ribonuclease pancreatic from nilgai.  
**rnp\_bovin:** ribonuclease pancreatic precursor from bovine.  
**rnp\_bubar:** ribonuclease pancreatic from domestic water buffalo.  
**rnp\_camdr:** ribonuclease pancreatic from dromedary.  
**rnp\_capca:** ribonuclease pancreatic from roe deer.  
**rnp\_caphi:** ribonuclease pancreatic from goat.  
**rnp\_cerel:** ribonuclease pancreatic from red deer.  
**rnp\_chese:** ribonuclease from snapping turtle.  
**rnp\_chibr:** ribonuclease pancreatic from chinchilla.  
**rnp\_choho:** ribonuclease pancreatic from Hoffmann's two-fingered sloth.  
**rnp\_conta:** ribonuclease pancreatic from brindled gnu.  
**rnp\_criilo:** ribonuclease pancreatic precursor from long-tailed hamster.  
**rnp\_damda:** ribonuclease pancreatic from fallow deer.  
**rnp\_damko:** ribonuclease pancreatic from topi.  
**rnp\_galmu:** ribonuclease pancreatic from cuis.  
**rnp\_gazth:** ribonuclease pancreatic from Thomson's gazelle.  
**rnp\_girca:** ribonuclease pancreatic from giraffe.  
**rnp\_hipam:** ribonuclease pancreatic from hippopotamus.  
**rnp\_horse:** ribonuclease pancreatic from horse.  
**rnp\_human:** ribonuclease pancreatic precursor from human.  
**rnp\_hydh:** ribonuclease pancreatic from capybara.  
**rnp\_hyscr:** ribonuclease pancreatic from crested porcupine.  
**rnp\_macru:** ribonuclease pancreatic from red kangaroo.  
**rnp\_mesau:** ribonuclease pancreatic from golden hamster.  
**rnp\_mouse:** ribonuclease pancreatic from mouse.  
**rnp\_myoco:** ribonuclease pancreatic from nutria.  
**rnp\_odovi:** ribonuclease pancreatic from virginia white-tailed deer.  
**rnp\_ondzi:** ribonuclease pancreatic from muskrat.  
**rnp\_pig:** ribonuclease pancreatic from pig.  
**rnp\_preen:** ribonuclease pancreatic from hanuman langur.  
**rnp\_progu:** ribonuclease pancreatic from casiragua.  
**rnp\_ranta:** ribonuclease pancreatic from reindeer.  
**rnp\_rat:** ribonuclease pancreatic precursor from rat.  
**rnp\_sheep:** ribonuclease pancreatic from sheep.  
**rnp\_spaeh:** ribonuclease pancreatic from Ehrenberg's mole rat.  
**rnp\_tauor:** ribonuclease pancreatic from eland.  
**rnpa\_cavpo:** ribonuclease pancreatic A from guinea pig.  
**rnpb\_cavpo:** ribonuclease pancreatic B from guinea pig.  
**rns\_bovin:** ribonuclease, seminal precursor from bovine.



RNKD_HUMAN	MVPKLFTSQI	CLLLLLGLLA	VEGSLHVKPP	QFTWAQWFET	QHINMTSQQ.
ECP_HUMAN	MVPKLFTSQI	CLLLLLGLMG	VEGSLHARPP	QFTRAQWFAI	QHISLNPPR.
RNKD_BOVIN	.....	.....	.....VPK	GLTKARWFEI	QHIQPRLLQ.
RNL4_BOVIN	.....	.....	.....	ZDRMYQRFLR	QHVDPDDETGG
RNL3_PIG	.....	.....	.....	ZDRMYQRFLR	QHVDPDATGG
RNP_CRILO	.....	.....	.VQPSLG...	KESAAMKFER	QHMDSTVATS
RNP_MESAU	.....	.....	.....	KESAAMKFER	QHMDSTVATS
RNP_ONDZI	.....	.....	.....	KETSAQKFER	QHMDSTGSSS
RNP_MOUSE	..MGLEKSLI	LFPLFFLLG	WVQPSLG...	RESAAQKFOR	QHMDPDGSSI
RNP_RAT	..MGLEKSLF	LFSLLVLVLG	WVQPSLGGES	RESSADKFKR	QHMDTEGPSK
RNP_HUMAN	.....	.....	....VQPSLG	KESRAKKFOR	QHMDSDSSPS
RNP_PREEN	.....	.....	.....	GESRAEKFOR	QHMDSGSSPS
RNP_HORSE	.....	.....	.....	KESPAKKFER	QHMDSGSTSS
RNP_CHOHO	.....	.....	.....	KETAAMKFOR	QHMDSGSSLS
RNPA_CAVPO	.....	.....	.....	AESSAMKFER	QHVDSDGSSS
RNP_HYDHY	.....	.....	.....	AESSAMKFOR	QHVDSEGSSS
RNPB_CAVPO	.....	.....	.....	AESSAMKFOR	QHMDPEGPS
RNP_GALMU	.....	.....	.....	AESSAMKFOR	QHMDSDGHPD
RNP_PROGU	.....	.....	.....	KESSAKKFOR	QHIDSSGSPS
RNP_MYOCO	.....	.....	.....	SESSAKKFER	QHMDSRGSPS
RNP_CHIBR	.....	.....	.....	KESSAMKFOR	QHMDSSGSPS
RNP_HYSCR	.....	.....	.....	KESSAMKFER	QHMDSSGSPS
RNP_CEREL	.....	.....	.....	KESAAAKFER	QHMDPSTSSA
RNP_DAMDA	.....	.....	.....	KESAAAKFER	QHMDPSMSSA
RNP_RANTA	.....	.....	.....	KESAAAKFER	QHMDPSPSSA
RNP_CAPCA	.....	.....	.....	KESAAAKFER	QHMDPSPSSA
RNP_ALCAA	.....	.....	.....	KESAAAKFER	QHMDPSASSI
RNP_TAUOR	.....	.....	.....	KETAAAKFER	QHMDSSSTSSA
RNP_BOSTR	.....	.....	.....	KETAAAKFER	QHMDSSSTSSA
RNP_BOVIN	...MALKSL	VLLSLLVLVL	LLVRVQPSLG	KETAAAKFER	QHMDSSSTSSA
RNP_BUBAR	.....	.....	.....	KETAAAKFOR	QHMDSSSTSSA
RNP_SHEEP	.....	.....	.....	KESAAAKFER	QHMDSSSTSSA
RNP_AEPME	.....	.....	.....	KESAAAKFER	QHMDSSSTSSA
RNP_CONTA	.....	.....	.....	KESAAAKFER	QHMDSSSTSSA
RNP_GAZTH	.....	.....	.....	KESAAAKFZR	ZHBSSTSSA
RNP_CAPHI	.....	.....	.....	KESAAAKFER	QHMDSSSTSSA
RNP_ANTAM	.....	.....	.....	KETAAAKFER	QHIDSNPSSV
RNP_GIRCA	.....	.....	.....	KESAAAKFER	QHIDSSTSSV
RNP_DAMKO	.....	.....	.....	KESAAAKFZR	ZHBSSTSSA
RNS_BOVIN	...MALKSL	VVLPLLVLVL	LLVRVQPSLG	KESAAAKFER	QHMDSGNSPS
RNP_HIPAM	.....	.....	.....	KETAEEKFOR	QHMDTSSSLS
RNP_PIG	.....	.....	.....	KESPAKKFOR	QHMDPDSSSS
RNP_BALAC	.....	.....	.....	RESPAMKFOR	QHMDSGNSPG
RNP_CAMDR	.....	.....	.....	SETAAEKFER	QHMDSYSSSS
RNP_SPAEH	.....	.....	.....	KESSAEKFLR	QHVDSIDSPG
RNP_MACRU	.....	.....	.....	.ETPAEKFOR	QHMDTEHSTA
RNP_ODOVI	.....	.....	.....	KETAAAKFER	QHMDP.APAA
ANGI_HUMAN	....MVMGL	GVLLLVFVLG	LGLTPPTLAQ	DNSRYTHFLT	QHYD.AKPKG
ANGI_MOUSE	....MAISP	GPLFLIFVLG	LVVIPPTLAQ	DDRYTKFLT	QHHD.AKPKG
ANGI_BOVIN	.....	.....	.....AQ	DDRYIHFLT	QHYD.AKPKG
RNP_CHESE	.....	.....	.....	.ETRYEKFLR	QHVDYPKSSA
ANGI_CHICK	.....MA	MSSLWWTAIL	LLALTVSMCY	GVPTYQDFLR	THVDFPKTSF
LECS_RANJA	.....	.....	.....	..ZNWAKFQE	KHI.....P



RNKD_HUMAN	.....CTNA	MQVINN.YQR	RCKNQNTFLL	TTFANVVNVC	GNPNMTCPSN
ECP_HUMAN	.....CTIA	MRAINN.YRW	RCKNQNTFLR	TTFANVVNVC	GNQSIRCPHN
RNKD_BOVIN	.....CNKA	MSGVNN.YTQ	HCKPENTFLH	NVFQDVTAVC	DMPNIICKNG
RNL4_BOVIN	NDS..YCNLM	MQRRKM.TSH	QCKRFNTFIH	EDLWNIRSIC	STTNIQCKNG
RNL3_PIG	NDA..YCNLM	MQRRKM.TSH	YCKRFNTFIH	EDIWNIRSIC	STSNIQCKNG
RNP_CRIL0	S.SPTYCNQM	MKRRNM.TQG	QCKPVNTFVH	ESLADVHAVC	SQENVKCKNG
RNP_MESAU	S.SPTYCNQM	MKRRNM.TQG	YCKPVNTFVH	ESLADVHAVC	SQENVACKNG
RNP_ONDZI	S.SPTYCNQM	MKRREM.TQG	YCKPVNTFVH	EPLADVQAVC	SQENVTCKNG
RNP_MOUSE	N.SPTYCNQM	MKRRDM.TNG	SCKPVNTFVH	EPLADVQAVC	SQENVTCKNR
RNP_RAT	S.SPTYCNQM	MKRQGM.TKG	SCKPVNTFVH	EPLEDVQAIC	SQGQVTCKNG
RNP_HUMAN	S.SSTYCNQM	MRRRNM.TQG	RCKPVNTFVH	EPLVDVQNV	FQEKVTCKNG
RNP_PREN	S.SSTYCNQM	MKLRNM.TQG	SCKSVNTFVH	EPLVDVQNV	FQEKVTCKNG
RNP_HORSE	S.NPTYCNQM	MKRRNM.TQG	WCKPVNTFVH	EPLADVQAIC	LQKNITCKNG
RNP_CHOHO	S.SSDYCNKM	MKVRNM.TQE	SCKPVNTFVH	ESLQDVQAVC	FQENVTCKNG
RNPA_CAVPO	S.NANYCNEM	MKKREM.TKD	RCKPVNTFVH	EPLAEVQAVC	SQRNVSCCKNG
RNP_HYDHY	S.NANYCNEM	MVRRKM.TQD	RCKPVNTFVH	EPLADVQAVC	FQKNVPCKNG
RNPB_CAVPO	N.SSNYCNVM	MIRRNM.TQG	RCKPVNTFVH	ESLADVQAVC	FQKNVLCKNG
RNP_GALMU	T.NTNYCNEM	MVRRSM.TQG	RCKPVNTFVH	EPLAVQAVC	SQKNVPCKNG
RNP_PROGU	T.NPNYCNAM	MKSRNM.TQE	RCKPVNTFVH	EPLADVQAVC	FQKNVPCKNG
RNP_MYOCO	T.NPNYCNEM	MKSRNM.TQG	RCKPVNTFVH	EPLADVQAVC	FQKNVLCKNG
RNP_CHIBR	T.NANYCNEM	MKGRNM.TQG	YCKPVNTFVH	EPLADVQAVC	FQKNVPCKNG
RNP_HYSCR	S.NSNYCNEM	MRRRNM.TQD	RCKPVNTFVH	EPLADVRAVC	FQKNVACKNG
RNP_CEREL	S.SSNYCNQM	MQSRKM.TQD	RCKPVNTFVH	ESLADVQAVC	FQKNVACKNG
RNP_DAMDA	S.SSNYCNQM	MQSRKM.TQD	RCKPVNTFVH	ESLADVQAVC	FQKNVACKNG
RNP_RANTA	S.SSNYCNQM	MQSRDL.TQD	RCKPVNTFVH	ESLADVQAVC	FQKNVACKNG
RNP_CAPCA	S.SSNYCNQM	MQSRNL.TQD	RCKPVNTFVH	ESLADVQAVC	FQKNVICKNG
RNP_ALCAA	S.SSNYCNQM	MQSRNL.TQD	RCKPVNTFVH	ESLADVQAVC	FQKNVACKNG
RNP_TAUOR	S.SSNYCNQM	MKSRDM.TKD	RCKPVNTFVH	ZSLABVZAVC	SZKBVACKBG
RNP_BOSTR	S.SSNYCNQM	MKSRSM.TQN	RCKPVNTFVH	ZSLABVQAVC	SZKBVACKBG
RNP_BOVIN	S.SSNYCNQM	MKSRNL.TKD	RCKPVNTFVH	ESLADVQAVC	SQKNVACKNG
RNP_BUBAR	S.SSNYCNQM	MKSRNM.TSD	RCKPVNTFVH	ESLADVQAVC	SQENVACKNG
RNP_SHEEP	S.SSNYCNQM	MKSRNL.TQD	RCKPVNTFVH	ESLADVQAVC	SQKNVACKNG
RNP_AEPME	S.SSNYCNQM	MKSRNL.TQS	RCKPVNTFVH	ESLADVQAVC	SQKNVACKNG
RNP_CONTA	S.SSNYCNQM	MKSRNL.TQD	RCKPVNTFVH	EPLADVQAVC	SQKNVACKNG
RNP_GAZTH	S.SSNYCNQM	MKSRNL.TQD	RCKPVNTFVH	ESLADVQAVC	SQKNVQCKNG
RNP_CAPHI	S.SSNYCBZM	MKSRNL.TQD	RCKPVNTFVH	ESLADVZAVC	SZKNVACKNG
RNP_ANTAM	S.SSNYCNQM	MKSRNL.TQG	RCKPVNTFVH	ESLADVQAVC	SQKNVACKNG
RNP_GIRCA	S.SSNYCNQM	MTSRNL.TQD	RCKPVNTFVH	ESLADVQAVC	SQKNVACKNG
RNP_DAMKO	S.SSBYCBZM	MKSRNL.TQD	RCKPVNTFVH	ZSLABVZAVC	SZKBVACKBG
RNS_BOVIN	S.SSNYCNLM	MCCRKM.TQG	KCKPVNTFVH	ESLADVKAVC	SQKKVTCKNG
RNP_HIPAM	N.DSNYCNQM	MVRRNM.TQD	RCKPVNTFVH	ESEADVKAVC	SQKNVTCKNG
RNP_PIG	N.SSNYCNLM	MSRRNM.TQG	RCKPVNTFVH	ESLADVQAVC	SQINVNCKNG
RNP_BALAC	N.NPNYCNQM	MMRRKM.TQG	RCKPVNTFVH	ESLEDVKAVC	SQKNVLCKNG
RNP_CAMDR	S.NSNYCNQM	MKRREM.TDG	WCKPVNTFIH	ESLEDVQAVC	SQKSVTCKNG
RNP_SPAEH	NLSPTYCNQM	MLRRNM.TKG	SCKPVNTFVH	EPLKDIKAVC	FQENVTCKNG
RNP_MACRU	S.SSNYCNLM	MKARDM.TSG	RCKPLNTFIH	EPKSVVDVAVC	HQENVTCKNG
RNP_ODOVI	AXXQNYCNQM	M.....	.....	.....	.....
ANGI_HUMAN	RDDR.YCESI	MRRRGL.TSP	.CKDINTFIH	GNKRSIKAIC	ENKNGNPHRE
ANGI_MOUSE	RDDR.YCERM	MKRRSL.TSP	.CKDVNTFIH	GNKSNIKAIC	GA.NGSPYRE
ANGI_BOVIN	RNDE.YCFNM	MKNRRL.TRP	.CKDRNTFIH	GNKNDIKAIC	EDRNGQPYRG
RNP_CHESE	PDSRTYCNQM	MQRRGM.TSP	VCKFTNTFVH	ASAASITTV	GS.GGTPASG
ANGI_CHICK	PNIAAYCNVM	MVRRGINVHG	RCKSLNTFVH	TDPRNLNTLC	INQPNRALRT
LECS_RANJA	NTSNINCNTI	MDKSIYIVGG	QCKERTFII	SSATTVKAIC	S.....G

RNKD_HUMAN	KTRKNCHHSG	SQVPLIHCNL	TTPSPQINIS	CRYAQTPANM	FYIVACDNRD
ECP_HUMAN	RTLNNCHRSR	FRVPLLHCDL	INPGAQINIS	CRYADRPGRR	FYVVACDNRD
RNKD_BOVIN	R..HNCHQSP	KPVNLTQCNF	I...AGRYPD	CRYHDDAQYK	FFIVACDPPQ
RNL4_BOVIN	QM..NCHEG.	.VVRVTDCRE	T..GSSRAPN	CRYRAKASTR	RVVIACEGN.
RNL3_PIG	QM..NCHEG.	.VVKVTDCRE	T..GSSRAPN	CRYRAMASTR	RVVIACEGN.
RNP_CRILO	KS..NCYKSH	SALHITDCRL	K..GNAKYPN	CDYQTSQHOK	HIIVACEGN.
RNP_MESAU	KS..NCYKSH	SALHITDCRL	K..GNAKYPN	CDYQTSQLQK	QIIVACEGN.
RNP_ONDZI	NS..NCYKSR	SALHITDCRL	K..GNSKYPN	CDYQTSQLQK	QVIVACEGS.
RNP_MOUSE	KS..NCYKSS	SALHITDCHL	K..GNSKYPN	CDYKTTQYQK	HIIVACEGN.
RNP_RAT	RN..NCHKSS	STLRITDCRL	K..GSSKYPN	CDYTTTDSQK	HI I IACDGN.
RNP_HUMAN	QG..NCYKSN	SSMHITDCRL	T..NGSRYPN	CAYRTSPKER	HIIVACEGS.
RNP_PREEN	QT..NCFKSN	SRMHITECRL	T..NGSKYPN	CAYGTSPKER	HIIVACEGS.
RNP_HORSE	QS..NCYQSS	SSMHITDCRL	T..SGSKYPN	CAYQTSQKER	HIIVACEGN.
RNP_CHOHO	QQ..NCHQSR	SNMHITDCRQ	T..SGSKYPN	CLYQTSNMNR	HI I IACEGN.
RNPA_CAVPO	QT..NCYQSY	SSMHITECRL	T..SGSKFPN	CSYRTSQAQK	SIIVACEGK.
RNP_HYDHY	QT..NCYQSY	SSMHITDCRV	T..SNSKFPD	CSYRTTQAQK	SIVVACEGN.
RNPB_CAVPO	QT..NCYQSY	SRMRITDCRV	T..SSSKFPN	CSYRMSQAQK	SIIVACEGD.
RNP_GALMU	QT..NCYQSH	SSMRITDCRV	T..SSSKYPN	CSYRMTQAQK	SIIVACEGT.
RNP_PROGU	QS..NCYEST	SNMHITDCRL	T..SNSKFPD	CLYRTSQEEK	SIIVACEGN.
RNP_MYOCO	QT..NCYQSN	SNMHITDCRV	T..SNSDYPN	CSYRTSQEEK	SIVVACEGN.
RNP_CHIBR	QS..NCYQSN	SNMHITDCRL	T..SNSKYPN	CSYRTSRENK	GIIVACEGN.
RNP_HYSCR	QT..NCYQSN	SLMHITDCRV	T..GSSKYPD	CSYGMSQLER	SIVVACEGS.
RNP_CEREL	QS..NCYQSN	SAMHITDCRE	S..GNSKYPN	CVYKATQAEK	HIIVACEGN.
RNP_DAMDA	QS..NCYQSN	SAMHITDCRE	S..GNSKYPN	CVYKATQAEK	HIIVACEGN.
RNP_RANTA	QS..NCYQSN	SAMHITDCRE	T..GSSKYPN	CVYKTTQAEK	HIIVACEGN.
RNP_CAPCA	QS..NCYQSN	SAMHITDCRE	S..GNSKYPN	CVYKTTQAEK	HIIVACEGN.
RNP_ALCAA	QS..NCYQSN	SAMHITDCRE	S..GNSDYPN	CVYKTTQAEK	HIIVACEGN.
RNP_TAUOR	ZT..BCYZSY	STMSITBCRZ	T..GSSKYPB	CAYKTTZAZK	HIIVACZGB.
RNP_BOSTR	ZT..BCYZSY	STMSITBCRZ	T..GSSKYPB	CAYTTTZAKK	HIIVACZGB.
RNP_BOVIN	QT..NCYQSY	STMSITDCRE	T..GSSKYPN	CAYKTTQANK	HIIVACEGN.
RNP_BUBAR	QT..NCYQSY	STMSITDCRE	T..GSSKYPN	CAYKTTQANK	HIIVACEGN.
RNP_SHEEP	QT..NCYQSY	STMSITDCRE	T..GSSKYPN	CAYKTTQAEK	HIIVACEGN.
RNP_AEPME	QT..NCYQSY	STMSITDCRE	T..GSSKYPN	CAYKTTQAKK	HIIVACEGN.
RNP_CONTA	QT..NCYQSY	STMSITDCRE	T..GSSKYPN	CAYKATQAKK	HIIVACEGN.
RNP_GAZTH	QT..NCYQSY	STMSITDCRE	T..GSSKYPN	CAYKTTQAQK	HIIVACEGN.
RNP_CAPHI	ZT..BCYQSY	STMSITBCRZ	T..GSSKYPN	CAYKTTQAEK	HIIVACZGB.
RNP_ANTAM	QT..NCYQSY	STMSITDCRE	T..GSSKYPN	CAYKTTQAKK	HIIVACEGN.
RNP_GIRCA	QT..NCYQSY	SAMSITDCRE	T..GNSKYPN	CAYQTTQAEK	HIIVACEGN.
RNP_DAMKO	ZT..BCYZSY	STMSITBCRZ	T..GSSKYPB	CAYKTTQAKK	HIIVACZGB.
RNS_BOVIN	QT..NCYQSK	STMRTDCRE	T..GSSKYPN	CAYKTTQVEK	HIIVACGGK.
RNP_HIPAM	QT..NCYQSN	STMHITDCRE	T..GSSKYPN	CAYKTSQLQK	HIIVACEGD.
RNP_PIG	QT..NCYQSN	STMHITDCRQ	T..GSSKYPN	CAYKASQEQK	HIIVACEGN.
RNP_BALAC	RT..NCYESN	STMHITDCRQ	T..GSSKYPN	CAYKTSQKEK	HIIVACEGN.
RNP_CAMDR	QT..NCHQSS	TTMHITDCRE	T..GSSKYPN	CAYKASNLK	HI I IACEGN.
RNP_SPAEH	NS..NCYKSH	ASLHITDCRT	I..SSSKYPD	CAYKTSQEQK	HIIVACEGD.
RNP_MACRU	RT..NCYKSN	SRLSITNCRQ	T..GASKYPN	CQYETSNLNK	QIIVACEGQ.
RNP_ODOVI	.....	.....	.....	.....	.....
ANGI_HUMAN	NL..R..ISK	SSFQVTTCKL	H..GGSPWPP	CQYRATAGFR	NVVVACENG.
ANGI_MOUSE	NL..R..MSK	SPFQVTTCKH	T..GGSPRPP	CQYRASAGFR	HVVIACENG.
ANGI_BOVIN	DL..R..ISK	SEFQITICKH	K..GGSSRPP	CRYGATEDSR	VIVVGCENG.
RNP_CHESE	DL..R..DSN	ASFALTTCL	Q..GGSQTPN	CPYNADASTQ	RIRIACVGG.
ANGI_CHICK	.....TQ	QQLPVTDCKL	I....RSHPT	CSYTGNOFNH	RVRVGCWGG.
LECS_RANJA	ASTNRNVLST	TRFQLNTCIR	SATAPRPCP.	..YNSRTETN	VICVKCENR.

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RNKD_HUMAN	QRRDPPQYPV	VPVHLDRII.	....
ECP_HUMAN	.PRDSPRYPV	VPVHLDTTI.	....
RNKD_BOVIN	..KTDPYHL	VPVHLDKYF.	....
RNL4_BOVIN	.....PE	VPVHFDK...	....
RNL3_PIG	.....PE	VPVHFDK...	....
RNP_CRILO	.....PF	VPVHFDATV.	....
RNP_MESAU	.....PF	VPVHFDASV.	....
RNP_ONDZI	.....PF	VPVHFDASV.	....
RNP_MOUSE	.....PY	VPVHFDATV.	....
RNP_RAT	.....PY	VPVHFDASV.	....
RNP_HUMAN	.....PY	VPVHFDASVE	DST.
RNP_PREEN	.....PY	VPVHFDSDVE	DST.
RNP_HORSE	.....PY	VPVHFDASVE	VST.
RNP_CHOHO	.....PY	VPVHFDASVE	DST.
RNPA_CAVPO	.....PY	VPVHFDNSV.	....
RNP_HYDHY	.....LY	VPVHFDASVE	PST.
RNPB_CAVPO	.....PY	VPVHFDASVE	PST.
RNP_GALMU	.....PS	VPVHFDATV.	....
RNP_PROGU	.....PY	VPVHFDASVA	ASA.
RNP_MYOCO	.....PY	VPVHFDASVA	ASA.
RNP_CHIBR	.....PY	VPVHFDASV.	....
RNP_HYSCR	.....PY	VPVHFDASVG	PST.
RNP_CEREL	.....PY	VPVHFDASV.	....
RNP_DAMDA	.....PY	VPVHFDASV.	....
RNP_RANTA	.....PY	VPVHFDASV.	....
RNP_CAPCA	.....PY	VPVHFDASV.	....
RNP_ALCAA	.....PY	VPVHFDASV.	....
RNP_TAUOR	.....PY	VPVHFBASV.	....
RNP_BOSTR	.....PY	VPVHFBASV.	....
RNP_BOVIN	.....PY	VPVHFDASV.	....
RNP_BUBAR	.....PY	VPVHYDASV.	....
RNP_SHEEP	.....PY	VPVHFDASV.	....
RNP_AEPME	.....PY	VPVHFDASV.	....
RNP_CONTA	.....PY	VPVHFDASV.	....
RNP_GAZTH	.....PY	VPVHFDASV.	....
RNP_CAPHI	.....PY	VPVHFDASV.	....
RNP_ANTAM	.....PY	VPVHYDASV.	....
RNP_GIRCA	.....PY	VPVHYDASV.	....
RNP_DAMKO	.....PY	VPVHFBASV.	....
RNS_BOVIN	.....PS	VPVHFDASV.	....
RNP_HIPAM	.....PY	VPVHYDASV.	....
RNP_PIG	.....PP	VPVHFDASV.	....
RNP_BALAC	.....PY	VPVHFDNSV.	....
RNP_CAMDR	.....PY	VPVHFDASV.	....
RNP_SPAEH	.....PF	VPVHYDASV.	....
RNP_MACRU	.....Y	VPVHFDAYV.	....
RNP_ODOVI	.....	.....	....
ANGI_HUMAN	.....	LPVHLDQSIF	RRP.
ANGI_MOUSE	.....	LPVHFDESFF	SL..
ANGI_BOVIN	.....	LPVHFDESFI	TRPH
RNP_CHESE	.....	LPVHYDKSI.	....
ANGI_CHICK	.....	LPVHLDGTFP	....
LECS_RANJA	.....	LPVHFAGIGR	C...

□ active site residues.

□ region 65-72 of RNase or region 97-106 of the precursor form of RNase.