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**STUDIES OF A RAT LIVER**  
**RIBONUCLEASE INHIBITOR PROTEIN GENE**

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Department of Biochemistry, University of Glasgow.

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## ABBREVIATIONS

The abbreviations used in this thesis are in agreement with recommendations of the editors of the Biochemical Journal (Biochem. J. (1983) 209, 1-27), except the following:

SDS	Sodium dodecyl sulphate.
hnRNA	Heterogenous RNA.
bp	Base pairs.
SSC	Standard saline citrate.
RNase A	Pancreatic ribonuclease A
BSA	Bovine serum albumin.
cpm	counts per minute.
ELISA	Enzyme linked immunosorbant assay.
TEMED	N, N, N', N'-tetramethylene diamine.
poly (A) <sup>+</sup>	Polyadenylated mRNA species.
SSPE	Standard saline phosphate EDTA.
IPTG	isopropyl $\beta$ -D thiogalactopyranoside.
Xgal	5-bromo-4-chloro-3-indolyl- $\beta$ -D galactoside

## SUMMARY

The cytoplasmic ribonuclease inhibitor-ribonuclease system has been detected in a wide variety of tissues and species and is thought to play an essential role in the regulation of RNA metabolism and hence protein synthesis. This hypothesis is based on the observation that tissues with high levels of RNA and protein synthesis are associated with correspondingly high levels of free inhibitor and *vice versa*. However, the precise nature of this regulatory function is unknown. Therefore, the aim of this project was to isolate cDNA clones of ribonuclease inhibitor from a rat liver library in the expression vector,  $\lambda$ gt11 using polyclonal antiserum, to characterise the resultant clones and to use the clones to determine the extent to which ribonuclease inhibitor activities are controlled at the level of gene expression

The initial task was to obtain a high titre, highly specific antiserum to be used as a probe in the screening of the library. Thus, inhibitor protein was purified from rat liver and used to raise polyclonal antiserum in rabbits. Analysis of the immune serum by western blotting revealed that, in addition to recognising purified ribonuclease inhibitor, the antiserum also recognised several other unknown proteins from a preparation of rat liver cytoplasm. These contaminating antibodies were adsorbed from the immune serum by affinity chromatography. The adsorbed antiserum was analysed by western blotting which demonstrated that it now recognised only one protein from rat liver cytoplasm which corresponded to the ribonuclease inhibitor. Polyclonal

antiserum also contains antibodies which bind to antigens produced by *E.coli*. This binding activity may result in a high background when screening the library which may make the identification of positive clones difficult. These *E.coli* antibodies were removed from the immune serum by adsorption against an *E.coli* crude cell lysate. To further reduce the possibility of a high background, IgG was purified from the immune serum and used to screen the library.

The rat liver cDNA library in the phage expression vector,  $\lambda$ gt11 was kindly provided by Richard Hynes. The cDNA inserts are cloned into the EcoR1 site of the  $\beta$ -galactosidase gene and, when in the correct frame and orientation, give rise to fusion proteins comprising a large portion of  $\beta$ -galactosidase fused to protein sequences encoded by the cDNA insert. Thus, cDNA clones may be isolated by screening large numbers of recombinant phage with antibodies raised against the protein of interest. Initial plaque screening of 300,000 recombinants with the purified IgG identified five putative ribonuclease inhibitor clones. The clones were taken through several rounds of purification and only three of the original five positives continued to produce a positive signal. To characterise the fusion proteins from the three clones (denoted  $\lambda$ RLRI 3,  $\lambda$ RLRI 7 and  $\lambda$ RLRI 8), lysogens were prepared and IPTG-induced crude cell lysates were analysed by SDS polyacrylamide gel electrophoresis. The relative molecular mass ( $M_r$ ) of the fusion proteins was approximately 127,000 Daltons, although the fusion protein of  $\lambda$ RLRI 7 appeared to be slightly larger. The fusion proteins were further characterised by

western blotting of the crude cell lysates. This revealed that  $\lambda$ RLRI 7 and  $\lambda$ RLRI 8 produced immunopositive fusion proteins whereas  $\lambda$ RLRI 3 did not.

At this stage in the project, there was no published amino acid or nucleotide sequence data of ribonuclease inhibitor and hence a comparison of the nucleotide sequence of the cDNA inserts with known sequence was impossible. To prove that the clones encoded ribonuclease inhibitor, peptide mapping of purified ribonuclease inhibitor and the fusion proteins was carried out and the resultant peptides analysed by western blotting. It was hoped that similar peptide maps would be obtained which would suggest that the clones encoded ribonuclease inhibitor. However, the results obtained were irreproducible and hence inconclusive.

To aid in the characterisation of the clones, some amino acid sequence data of ribonuclease inhibitor was obtained. Tryptic and chymotryptic digests of the inhibitor were subjected to HPLC analysis and six of the resultant peptides were taken to Aberdeen University to be sequenced on their facilities. Only one of the peptides provided sequence data and a polypeptide of eleven amino acids was obtained.

Meanwhile, the cDNA inserts of  $\lambda$ RLRI 7 and  $\lambda$ RLRI 8 were subcloned into the plasmid vector, pTZ and nucleotide sequence was obtained using the dideoxy chain termination method of Sanger. The sequence of the two inserts was identical with pRLRI 7 being approximately 65 nucleotides longer than pRLRI 8. This corresponds to the previous data where the fusion protein for  $\lambda$ RLRI 7 was slightly larger than that for  $\lambda$ RLRI 8.

At this time, nucleotide and amino acid sequence data for the human placental ribonuclease inhibitor was published. When compared to the nucleotide and predicted amino acid sequences of pRLRI 7 and pRLRI 8, there was no apparent homology. The full-length human placental cDNA clone was kindly sent by Professor Bert Vallee and Southern blot analysis revealed that there was no cross-hybridisation between the rat liver clones and the human placental clone. Northern blotting analysis showed that the human clone does not cross-hybridise with rat liver RNA which suggests that there may be little homology between the human ribonuclease inhibitor and the rat liver inhibitor.

Preliminary data on the expression of the isolated rat clones showed that they are expressed in rat liver, kidney, brain and heart.



**CHAPTER ONE**

**INTRODUCTION**

## THE REGULATION OF mRNA DEGRADATION

### 1.1 INTRODUCTION

The steady state level of cytoplasmic mRNA is dependent upon its rate of synthesis and degradation. Until recently, studies on the mechanisms regulating the levels of mRNA have concentrated on the transcriptional control of mRNA synthesis (reviewed by Maniatis *et al.*, 1987). However the metabolism of mRNA, which includes the processing of nuclear RNA, export from the nucleus and mRNA degradation, has emerged as a major control mechanism in regulating the level of mRNA in the cytoplasm and is probably as important as transcription in the control of gene expression. The factors controlling the rate of degradation of mRNA are now beginning to be elucidated (for reviews see Raghow, 1987; Brawerman, 1987; Cleveland, 1988; Hunt, 1988; Marzluff and Pandey, 1988 and Brawerman, 1989) and are described in some detail in this introduction. Of obvious importance are the ribonucleases which carry out the degradation of RNA and how they are regulated within the cell. This introduction describes the mammalian ribonucleases and their association with the endogenous ribonuclease inhibitor and how the ribonuclease:inhibitor complex may play a role in the regulation of mRNA turnover.

### 1.2 mRNA TURNOVER IN EUKARYOTES

Until recently, the rate of synthesis of mRNA was assumed to be the major determinant of the steady state level of mRNA in the eukaryotic cell. However, when the transcription rates of several constitutively expressed (housekeeping) genes in mouse L cell fibroblasts were

measured (Carneiro and Schubler, 1984), it was found that although some genes were transcribed at relatively low rates, their mRNAs were abundant whereas some genes with a high rate of transcription had mRNAs which were rare. In each case, the steady state level of a mRNA correlated with its stability i.e. the more stable the mRNA, the more abundant it was. Cellular mRNA levels actually represent a balance between rates of synthesis of nuclear RNA, processing and export from the nucleus and rates of cytoplasmic mRNA turnover (stability/degradation of mRNA).

The rates of mRNA turnover vary to a wide extent in eukaryotes. The half-lives of some mRNAs are extremely short e.g. the proto-oncogene, *c-fos* is degraded at a very high rate with a half-life of 30 minutes (Greenberg *et al.*, 1986) whereas other mRNAs are more stable with half-lives that span several hours or even days e.g. globin mRNA (Bastos *et al.*, 1977). The rate at which a mRNA molecule is degraded controls the speed with which changes in the transcription rate are reflected in its cytoplasmic level e.g. a mRNA with a short half-life responds rapidly to a change in the rate of transcription whereas a mRNA with a longer half-life responds more slowly to a change in transcription rate. Thus the turnover of mRNA is now considered to be a major control point in the regulation of gene expression.

### **1.2.1 CONTROL OF mRNA TURNOVER BY ENDOGENOUS AND EXOGENOUS FACTORS**

The rates at which individual genes are transcribed and the rate of turnover of specific mRNAs can be altered in response to certain endogenous and exogenous stimuli e.g.

changes in the cell-cycle or induction by hormones and growth factors.

Several constitutively expressed 'housekeeping' genes undergo changes in their degradation rate in response to changes in the cell cycle e.g. the mRNAs encoding thymidylate synthase (Ayusawa *et al.*, 1986) and histones (Graves *et al.*, 1987). The rates of transcription are observed to remain constant whereas the steady state levels of the mRNA vary considerably. The regulation of histone mRNA turnover is considered in more detail in Sections 1.2.3 to 1.2.6 inclusive.

Studies have been carried out to determine the effects of exogenous stimuli (hormones and growth factors) on the turnover of mRNA (for review see Shapiro *et al.*, 1987). Oestrogen is observed to have a dramatic effect on the stability of the mRNA encoding the egg-yolk precursor protein, vitellogenin (Brock and Shapiro, 1983). In oestrogen-treated cells, the half-life is 480 hours which represents a 30-fold change in the rate of mRNA turnover as compared to untreated cells. Oestrogen-stimulated stabilisation of vitellogenin mRNA appears to be selective as the half-life of the total mRNA in both treated and untreated cells is 16 hours (Baker and Shapiro, 1977)

The turnover of type 1 procollagen mRNA is affected by treatment with glucocorticoids and growth factors (Shapiro *et al.*, 1987). Addition of dexamethasone, a synthetic glucocorticoid, increases the degradation rate of this mRNA whereas transforming growth factor  $\beta$  (TGF- $\beta$ ) stabilises type 1 procollagen mRNA (Raghow *et al.*, 1987).

Specific stabilisation of cellular mRNAs by hormones and growth factors may be the result of covalent modifications of the mRNA during synthesis and processing in the nucleus which render the mRNAs resistant to degradation in the cytoplasm. The presence or

absence of a particular hormone or growth factor may cause the modifications to be irreversibly removed, thus altering the stability of the mRNA. Alternatively, the stability of mRNA may be regulated through reversible protein-RNA interactions in the cytoplasm. Initial studies carried out on oestrogen-stabilised vitellogenin mRNA (Brock and Shapiro, 1983) first revealed that there are sequences within the mRNA molecule which may confer a particular secondary structure on the molecule or act as recognition sites for nucleases and thus regulate its stability. The following sections describe the techniques which have been used to elucidate these regulatory sequences and outline in detail the known sequences within the mRNA molecule which are thought to play a role in the regulation of mRNA turnover.

### **1.2.2 TECHNIQUES USED TO STUDY mRNA TURNOVER**

Several techniques have been influential in determining how mRNA is regulated. The importance of a particular region of the mRNA molecule in RNA turnover has been demonstrated by the use of transfection experiments. Constructs of DNA are made in which regions of the mRNA are deleted, modified or transferred to other genes. The constructs are then transfected into cultured cells and the DNA transcribed into the modified mRNAs. The stability of the modified mRNAs are compared to the unmodified mRNA e.g. Fort *et al.*, (1987) deleted 130bp from the 3'non coding region of *c-fos*, transfected the modified gene into hamster fibroblasts and showed that the deletion stabilised the mRNA transcript. In this way sequences involved in the regulation of mRNA turnover are beginning to be elucidated.

A cell-free system has been devised by Ross and Kobs (1986) to

study the turnover of histone mRNA. In this system, mRNA turnover is studied by incubating mRNA with polyribosomes isolated from a cell lysate. This seems to be a valid method of studying mRNA turnover as the half-lives and degradation of mRNAs studied to date in both the cell-free system and *in vivo* appear to be similar. Using this system, the structural features which regulate the turnover of histone mRNAs are now beginning to be understood.

### 1.2.3 THE ROLE OF THE POLY(A) TAIL IN mRNA TURNOVER

The degradation of mRNA may be affected by the absence or presence of the poly(A) tail at the 3' end of the mRNA molecule. Removal of the poly(A) tail from certain mRNA species causes them to be degraded rapidly (Huez *et al.*, 1981) whereas addition of a poly(A) tail to other mRNAs increases their stability (Peltz *et al.*, 1987). Certain hormones which are known to stabilise specific mRNAs also induce elongation of the poly(A) tail (Paek and Axel, 1987) thus providing further evidence that there may be a correlation between the presence of a poly(A) tail and mRNA stability.

The absence of a poly(A) tail has been implicated in the regulation of histone mRNA. Most histone mRNAs do not possess a poly(A) tail (Adesnick and Darnell, 1972) and in many cell types are regulated as a function of the cell-cycle i.e. they are present at low levels during G<sub>1</sub> phase, accumulate during S phase and are degraded at the end of S phase (Robbins and Borun, 1967, Heintz *et al.*, 1983). Amphibian oocytes contain histone mRNAs which possess poly(A) tails (Levenson and Marcu, 1976). These mRNAs are stable during the period between oocyte development and fertilisation. The histone

mRNA loses its poly(A) tail during maturation of the oocyte to an egg and during embryogenesis and is then rapidly degraded (Hyman and Wormington, 1988). Also, histone mRNAs lacking poly(A) tails are rapidly degraded when injected into oocytes (Huez *et al.*, 1978). However, if a poly(A) tail is added to the histone mRNAs prior to micro-injection, then the mRNAs are stabilised (Huez *et al.*, 1978). These observations suggest that there is a correlation between the degradation of histone mRNA and the absence of a poly(A) tail.

*In vivo*, the poly(A) tail exists as a complex with a protein, the poly(A) binding protein (PABP) (Bergmann and Brewerman, 1977). It is thought that the PABP helps to stabilise mRNA by protecting the poly(A) segment from attack by nucleases. However, PABP does not protect every polyadenylated mRNA to the same extent which suggests that other sequences on the mRNA molecule may affect how efficiently PABP binds to the poly(A) tail (Brawerman, 1987). Wilson and Treisman (1988) demonstrated that in *c-fos* mRNA constructs lacking the AU-rich signal (see section 1.2.4), the poly(A) segment is more stable. Thus the destabilising effect of AU-rich sequences could be due to an interaction with the poly(A) tail/PABP complex.

Experiments carried out in a cell-free system by Brewer and Ross (1988) have shown that *c-myc* mRNA loses its poly(A) tail before the rest of the molecule is degraded. Thus it is possible that degradation of mRNA in eukaryotic cells is initiated by cleavage at the 3' terminus of the mRNA, resulting in deadenylation and consequent degradation of the molecule in a 3' to 5' direction.

#### 1.2.4 3'NON CODING REGION OF mRNA

Specific sequences in the 3'non coding region of certain mRNAs

seem to be important in determining their rates of degradation. Shaw and Kamen (1986) fused a 51 nucleotide AT-rich sequence from the 3'non coding region of a human lymphokine gene (GM-CSF) to a rabbit beta ( $\beta$ ) globin gene and found that the hybrid globin mRNA was degraded 10-30 times faster than a normal  $\beta$  globin transcript. Examination of the 3'non-coding regions of many transiently expressed genes, such as *c-myc*, *c-fos* and interferon, showed that they also contain this AT-rich sequence and therefore the degradation of these unstable mRNAs may be regulated by a common mechanism. At present the manner in which AU-rich sequences promote RNA degradation is unclear. It is possible that an endoribonuclease (or other binding protein that facilitates ribonuclease activity) cleaves specifically at AU-rich regions, thus leaving the mRNA susceptible to 3' exonucleolytic attack. However, it appears that this AU-rich sequence is not the only signal recognised by this putative AU-specific endoribonuclease as it is also present in the 3'non coding region of the mRNA of  $\beta$  globin, a transcript which is known to be highly stable. Also, when the AT-rich sequence of the GM-CSF gene was fused to a reporter mRNA, the transcript was stable in a monocytic tumour whereas an oncogene with a similar AT-rich region was rapidly degraded (Shyu *et al.*, 1989). This suggests that the AU-rich sequence of the 3'non coding region may interact with other sequences or structural elements of the mRNA to promote degradation of these mRNAs (see previous section for the interaction of AU-rich sequence and the poly(A) tail/PAPB complex).

The mRNA of *c-fos* contains an AU-rich sequence in the 3'non coding region (Van Straaten, 1983). However, when this sequence is deleted, the mRNA is still relatively unstable which suggests that the



turnover of *c-fos* mRNA is determined by more than one degradation signal. When the coding region of *c-fos* is substituted for the coding region of  $\beta$  globin mRNA, the hybrid transcript is unstable (Kabnick and Houseman, 1988). Thus, a further degradation signal appears to be present in the coding region for *c-fos* mRNA. Experiments carried out recently by Shyu et al., (1989) show that, rather than acting in concert, the degradation processes initiated by the two putative signals are different. The degradation of a transcript containing the *c-fos* AU-rich signal is decreased when transcription is inhibited whereas the degradation of a mRNA with the *c-fos* coding region is unaffected.

One approach to determine how the 3'non coding region affects the turnover of some mRNAs involves comparing the half-lives of closely-related, naturally occurring mRNAs in the same cell type. The mRNAs of human  $\beta$  globin and delta ( $\delta$ ) globin code for the protein components of two types of haemoglobin. The 5'non coding and coding regions of the mRNAs are very similar but the 3'non coding regions differ greatly. In human bone marrow cells, the mRNA of  $\delta$  globin is degraded 4-6 fold faster than the mRNA of  $\beta$  globin (Ross and Pizzaro, 1983). As the two molecules differ mainly in the 3'non coding region, the difference in turnover is probably due to sequences in the 3'non coding region of the  $\delta$  globin mRNA being more susceptible to degradation than those of the  $\beta$  globin mRNA.

As mentioned previously, cell cycle regulated histone mRNAs are degraded when DNA synthesis is arrested e.g. in G<sub>1</sub> phase of the cell cycle. Histone mRNAs are terminated by a highly conserved stem-loop structure at the 3'non coding region and it has been shown that the presence of this structure is required to couple the degradation of the mRNA with cessation of DNA replication (Levine *et al.*, 1987).

Degradation of H4 mRNA is apparently initiated by cleavage near the stem-loop structure and upon cessation of DNA replication, histone mRNA molecules lacking between 5 and 12 nucleotides at the 3' end are observed (Ross *et al.*, 1987). It is possible that during S phase, when there is a high demand for histones, a factor may bind to this stem-loop structure thus protecting it from nucleolytic attack. A similar stem-loop structure is also present in the 3' non coding region of the mRNA of the transferrin receptor and it is thought to promote degradation of this mRNA in the presence of iron (Muller and Kuhn, 1988).

#### 1.2.5 PROTEIN SYNTHESIS AND mRNA TURNOVER

Studies on the regulation of histone mRNAs (Graves *et al.*, 1987) and on the autoregulated stability of tubulin mRNA (Cleveland, 1988) have provided evidence that the regions of the mRNA which are translated into proteins contain signals for the regulation of mRNA degradation

The normal regulation of histone mRNA requires progression of the ribosomes to the correct termination codon. Graves *et al.*, (1987) mutated histone genes such that translation was initiated at the correct site but terminated prematurely. The half-lives of the mutated mRNAs were increased approximately 2-fold relative to normal histone mRNA transcripts. They also observed that the regulation of histone mRNA degradation requires that the distance between the highly conserved 3' stem-loop structure of histone mRNA and the translational termination codon must be no more than 300 bases. This suggests that the decay of the mRNA is initiated by a nuclease which may be associated with

the ribosomes and recognises some structural feature(s) of the 3' region of the histone mRNA. Termination of translation at the correct site would thus bring the nuclease to the target at the 3' terminus. Alternatively, the ribosome may in some way unwind the stem-loop structure thus exposing the recognition site to the nuclease.

In contrast to the situation outlined above, where premature termination of translation increases the stability of the histone mRNA, the absence of translating ribosomes beyond a certain region of the coding segment (due to premature termination) results in the rapid degradation of triosephosphate isomerase mRNA (Daar and Masquat, 1988). In this case, there does not appear to be a direct relationship between the length of the untranslated RNA and destabilisation. It is possible that premature termination of the triosephosphate mRNA causes a nuclease-sensitive site to be exposed to some nucleolytic activity and hence rapid degradation ensues.

Recently, it has been shown that there is a link between protein translation and mRNA turnover in the autoregulated decay of tubulin mRNA (Cleveland, 1988; Hunt, 1988 and references therein). The stability of tubulin mRNA is regulated by the level of unassembled tubulin subunits. Interestingly, the sequence or structure of the mRNA molecule itself is not necessary for autoregulation. Instead, the instability recognition element is the first four amino acids (Met-Arg-Glu Ile) of the newly synthesised (nascent) tubulin polypeptide chain. Furthermore, autoregulation requires that the tubulin mRNA must be bound to the polysome. A model for this autoregulated control has been proposed: as the level of unpolymerised tubulin subunits increases, tubulin interacts with the amino terminal of the nascent polypeptide chain after it emerges from the ribosome. In some way, as yet unknown, this protein-protein interaction mediates the subsequent

degradation of the mRNA molecule by either activating a ribosome-associated ribonuclease or by stalling the polysome, thus exposing a section of the RNA molecule to cytoplasmic ribonucleases.

Further evidence that translation of mRNA is linked to mRNA turnover comes from the observation that cycloheximide, an inhibitor of polypeptide chain elongation, causes stabilisation of many unstable mRNAs (Fort *et al.*, 1987). It has been suggested that this effect is due to loss of an unstable ribonuclease but there is no direct evidence to support this hypothesis. It is perhaps more likely that translational inhibitors modify the interaction between the ribosome and mRNA thus altering the susceptibility of the mRNA to degradation by nucleases.

#### 1.2.6 5'NON CODING REGION

It is also understood that sequences in the 5'non coding region can affect the stability of mRNA. Under normal circumstances, the mRNA of the proto-oncogene, *c-myc* is relatively unstable with a half-life of approximately 10 minutes (Dani *et al.*, 1984). However, in cancerous lymph node cells, the 5'non coding region of the *c-myc* mRNA is deleted. This truncated mRNA contains the entire coding region and the translated protein displays normal biological activity. However, deletion of the 5'non coding region increases the stability of the mRNA by 3 to 5-fold (Piechaczyk *et al.*, 1985; Ray *et al.*, 1987). At present, the mechanism by which the 5'non coding region affects the half-life of *c-myc* mRNA is unknown. It is possible that deletion of this region may affect the translation rate of the mRNA or it may affect the secondary structure of the mRNA. Although removal

of the 5'non coding region increases the stability of *c-myc* mRNA, fusion of this region with the chloramphenicol acetyl transferase (CAT) gene does not affect the stability of the CAT gene (Piechaczyk *et al.*, 1987). Thus, it seems likely that the destabilising 5'non coding region interacts with other regions of the *c-myc* mRNA (Saito *et al.*, 1983).

Further evidence that the 5'non coding sequence can affect the stability of mRNA comes from experiments carried out on the 5'non coding region of the human histone H3 mRNA. When the 5'non coding region of histone H3 mRNA is fused to  $\beta$  globin mRNA, the hybrid RNA is destabilised when DNA synthesis is blocked, thus suggesting that the 5'non coding region plays a role in histone H3 stability (Morris *et al.*, 1986). When the 5'non coding region of histone mRNA is replaced by that from the *Drosophila* heat shock protein 70 (hsp70) mRNA, the histone mRNA remains stable in the absence of DNA synthesis (Morris *et al.*, 1986). However, the 5'non coding region is not involved in the regulation of all of the histone mRNAs. The sequence that links mRNA stability to DNA synthesis in the histone H4 appears to be located in the 3'non coding region (Luscher *et al.*, 1985).

In general, most mRNAs are protected from degradation in a 5' to 3' direction by the presence of a cap at their 5' termini (Shatkin, 1976). In the absence of a cap or bound protein at the 5' end, mRNAs are degraded rapidly. It is unlikely, however, that the nuclease which degrades in a 5' to 3' direction is a major nuclease in selective mRNA degradation as most mRNA species are protected by a cap and also, most mRNAs are thought to be degraded from the 3'to the 5' end (Ross and Kobs, 1986; Ross *et al.*, 1987).

### **1.2.7 mRNA TURNOVER WITHIN THE NUCLEUS**

The previous sections have outlined the mechanisms which control the rate of degradation of mature mRNA molecules within the cytoplasmic fraction of the cell. There is also some evidence that degradation of pre-mRNA occurs within the nucleus (Vaessen et al., 1987). There are several stages in the processing of pre-mRNA to mature mRNA at which RNA degradation may occur e.g. the splicing of introns and polyadenylation. Caffarelli *et al.*, (1987) reported that the accumulation of mature RNA of *Xenopus laevis* ribosomal L1 protein is controlled at the level of splicing. In this case, when an excess of free L1 protein is present, an incompletely spliced precursor RNA accumulates. The two introns which have not been spliced out are specifically cleaved and the resultant truncated molecules are degraded. Similarly, two introns of the *Drosophila suppressor-of-white-apricot* gene are not removed after cellular blastoderm development, thus preventing the production of a functional mRNA (Chou *et al.*, 1987)

It is also possible that degradation of RNA may occur during transportation from the nucleus to the cytoplasm (Pena and Zasloff, 1987). However, as yet there have been no reports to support this possibility.

### **1.2.8 ENZYMES INVOLVED IN mRNA DEGRADATION**

Knowledge about the nucleases and other factors involved in the degradation of mRNA is limited. The cell-free system for investigating the turnover of histone mRNA (see Section 1.2.2)

revealed that a polysome-associated nuclease initiated degradation of histone mRNA by cleavage of the stem-loop structure at the 3' end of the RNA. The histone mRNA was then degraded in a 3' to 5' direction (Ross *et al.*, 1987). Hopefully, the establishment of this system will be furthered by the development of similar systems to investigate the degradation of other mRNA species and the role, if any, of non-polysome associated nucleases.

The presence of discrete, intermediate-sized products of mRNA breakdown suggests that a combined action of exo- and endonucleases may be responsible for completing the process of mRNA turnover e.g. an endonuclease which specifically recognises the AU-rich sequence of certain mRNA molecules could expose the 3' terminus of such mRNAs thus rendering them susceptible to degradation by a 3' exonuclease. However, no AU-rich sequence specific endonuclease has been identified as yet.

There is also very little information concerning the nature of possible factors which may regulate the action of the nucleases involved in mRNA degradation. Evidence that such factors may be intracellular molecules that are activated by appropriate signals is provided by studies carried out on interferon. Interferon induces the synthesis of several proteins, including 2'-5' oligoadenylate (2-5A) synthetase which synthesises the molecule 2-5A. This molecule activates an endoribonuclease which selectively degrades viral mRNAs (Baglioni *et al.*, 1984). Infection by *Herpes simplex* virus leads to non-specific degradation of most cellular mRNAs (Kwong and Frenbel, 1987). This degradation could result from the action of a viral gene product or by activation of a cellular nuclease.

Many questions have still to be answered concerning the nucleases and factors responsible for the degradation of mRNA e.g.

do mRNA-degrading ribonucleases act only on specific mRNAs or do they degrade non-specifically? Also, how are the ribonuclease activities regulated? Use of the cell-free system developed by Ross and Kobs (1986) will hopefully provide the answers to these questions in the near future.

### 1.3 CLASSIFICATION OF MAMMALIAN RIBONUCLEASES

It is evident from the preceding sections that one of the major functions of ribonucleases is the degradation of mRNA molecules. Ribonucleases are also required for a variety of other functions within the eukaryotic cell. Ribonucleases are necessary for processing of RNA molecules i.e. where one RNA molecule is converted to another e.g. the processing of heterogenous nuclear (hn) RNA to mature mRNA. This involves the addition of a cap,  $M^7G(5')ppp(5')-N$  at the 5' terminal end of the RNA (Shatkin, 1976; Shatkin, 1987), the addition of a poly(A) tail at the 3' terminus of the RNA molecule and the removal of intron transcripts (splicing).

Thus, there are many different types of ribonucleases to be found within the eukaryotic cell and they were classified by Sierakowska and Shugar in 1977 into two main types: endoribonucleases which act at specific sites within the RNA molecule yielding oligonucleotide fragments and exoribonucleases which digest RNA sequentially from the 3' or 5' terminus. Endogenous ribonuclease inhibitor is thought not to affect exoribonucleases and these enzymes are therefore omitted from the remainder of this discussion.

The endoribonucleases are further sub-divided into two groups: type I and type II endoribonucleases. Type I ribonucleases are



optimally active at alkaline or neutral pH and digest RNA via the formation of products with terminal pyrimidine nucleoside 2',3'-cyclic phosphates. Type I ribonucleases are sensitive to the ribonuclease inhibitor and are discussed in more detail in the following section. Type II endoribonucleases show optimal activity at pH5.0-6.0, digest RNA non specifically, are thermolabile and are found exclusively in the lysosomal fraction of mammalian cells. Type II endoribonucleases are also insensitive to the endogenous ribonuclease inhibitor (Blackburn and Moore, 1982)

Other nucleases which fall into neither the type I nor type II categories and are insensitive to the ribonuclease inhibitor are those which are involved in the processing of ribosomal RNA, heteronuclear RNA and transfer RNA, nucleases active towards double-stranded RNA, ribonuclease H which digests the RNA moiety of DNA:RNA hybrids and an endonuclease activated in response to interferon (Sierakowska and Shugar, 1977)

### 1.3.1 TYPE I ENDORIBONUCLEASES

Sierakowska and Shugar (1977) divided the type I endoribonucleases into two further subgroups: secretory and nonsecretory ribonucleases. The pancreatic ribonucleases are typical of the secretory class. In particular, bovine pancreatic ribonucleases A and B (RNase A and RNase B) have been well-characterised and the amino acid and nucleotide sequences are known (reviewed by Blackburn and Moore, 1982). A number of other secretory ribonucleases have been isolated from several sources e.g. the pancreas of human (Beintema *et al.*, 1984) and the pancreas of turtle (Beintema *et al.*, 1985a) and their amino acid sequences determined.

Comparison of these amino acid sequences with that of RNase A has revealed that they are all related. Also, a secretory ribonuclease has been isolated from bovine brain (Watanabe *et al.*, 1988) and shown to be 78% homologous to RNase A. Ribonucleases of the pancreatic type are secreted by the pancreas and salivary glands of all mammals and are found in the duodenal contents, kidney, serum and urine.

The other class of type I ribonucleases are the non secretory ribonucleases. These enzymes cleave most effectively at pH 6.5-7.0 and occur predominantly in liver, lung, spleen and leucocytes (Morita *et al.*, 1986). Ribonucleases of this class have also been isolated from lysosome-rich sources (Niwata *et al.*, 1985). A non secretory ribonuclease has been isolated from human urine and its amino acid sequence determined (Beintema *et al.*, 1988). Comparison of this sequence with that of non secretory ribonucleases from human liver (Sorrentino *et al.*, 1988) and bovine kidney (Irie *et al.*, 1988) shows that they are very closely related. Both the human urine and bovine kidney non secretory ribonuclease show approximately 30% homology with secreted mammalian ribonucleases. On the basis of this amino acid sequence data, mammalian secretory ribonucleases, human urine non secretory ribonuclease, turtle pancreatic ribonuclease and human angiogenin (see Section 1.4.6 for a brief discussion of this vascularising protein) have been grouped into a ribonuclease "superfamily".

The precise function of the non secretory ribonucleases is unclear. However, among these ribonucleases are a group of intracellular enzymes for which a regulatory role in RNA metabolism has been proposed. This hypothesis is based on the association of these ribonucleases with the endogenous ribonuclease inhibitor

(reviewed by Blackburn and Moore, 1982). Briefly, the ribonuclease-inhibitor complex may determine the rate of RNA degradation and thus the rate of protein biosynthesis. Tissues characterised by high rates of RNA synthesis have an excess of inhibitor present whereas tissues with high levels of RNA catabolism have an excess of free ribonuclease present. Also, "latent" ribonuclease activity, i.e. where the ribonuclease is bound to the inhibitor, can be activated depending on the physiological state of a particular cell. A detailed discussion of the ribonuclease inhibitor and its possible role in regulating RNA metabolism is presented in Section 1.4

### 1.3.2 ASSOCIATION OF RIBONUCLEASES WITH THE INHIBITOR IN VIVO

The intracellular ribonucleases associated with the endogenous inhibitor have not been thoroughly characterised due to the small quantities available within the cell. However, studies which were carried out revealed that more than one ribonuclease activity is bound to the inhibitor e.g. two peaks of latent ribonuclease activity were observed after anion-exchange chromatography of intact ribonuclease-inhibitor complexes from rat reticulocyte (Aoki *et al.*, 1981) and from mouse skeletal muscle (Little and Whittingham, 1981). A variety of cytoplasmic ribonucleases were partially purified from porcine thyroid and separated by gel filtration (Button *et al.*, 1982). The major ribonuclease activity was attributed to a species with a relative molecular mass (Mr) of 51,000 Daltons. The remainder of the activity corresponded to several species with Mr ranging from 13,000-28,000 Daltons. All of the ribonuclease activities were inhibited by the endogenous inhibitor. Blackburn *et al.*, 1982 proposed that the

ribonucleases associated with the inhibitor *in vivo* should resemble pancreatic RNase A in order to interact with the inhibitor and therefore it is surprising that the inhibitor inactivated the high molecular weight species purified by Button *et al.*, 1982.

More recently, several groups have purified ribonucleases that associate with the inhibitor *in vivo*. An inhibitor-associated cytoplasmic type I ribonuclease was first purified to homogeneity from rat liver by Kumagai *et al.*, (1983). The enzyme was isolated by obtaining the ribonuclease-inhibitor complex, inactivating the inhibitor with  $\text{CdCl}_2$ , then purifying the enzyme using ion-exchange chromatography. Sufficient amounts of the ribonuclease were obtained to allow characterisation of the enzyme. It was found to be a heat-stable protein with a Mr of 16,000 Daltons and a pH optimum of 7.5-8.0. The enzyme was found to be different from rat pancreatic ribonuclease in that it preferentially cleaved poly(C) over poly(U). The authors also reported the presence of a second, minor species of ribonuclease at an early stage in the purification procedure but this species was not further characterised.

Knowledge of the intracellular endoribonucleases was further enhanced when Brockdorff and Knowler (1987) purified two inhibitor-associated ribonuclease activities from rat liver and three activities from rat uterus using a more rapid purification procedure than Kumagai *et al.*, (1983). The main peak of activity from the rat liver (RLC I) was purified to homogeneity and characterised by determining its substrate preference and pH optima. This revealed that it is similar to the ribonuclease purified by Kumagai *et al.*, (1983). The other activity purified from rat liver (RLC II) by Brockdorff and Knowler differed from RLC I in that RLC II preferred yeast RNA to

poly(C) and the pH optima was different. Two of the activities purified from the rat uterus also preferentially degraded poly(C). The third activity (RUC III) degraded poly(U) preferentially.

Using a technique similar to that of Brockdorff and Knowler, two major and a number of minor species of ribonuclease activities were isolated from the human placenta by Blackburn (personal communication). The two major activities were purified to homogeneity and characterised. One activity was determined to be similar to human pancreatic ribonuclease while the other resembled the ribonuclease isolated from human liver.

#### **1.4 THE CYTOPLASMIC RIBONUCLEASE INHIBITOR**

In general, 90-95% of the type I endoribonucleases are present in the cell cytoplasm in an inactive 'latent' form due to their association with an endogenous inhibitor. The presence of this inhibitor in the high speed supernatant fraction prepared from guinea pig liver was first reported by Pirotte and Desreux in 1952. Most mammalian tissues have since been found to contain small amounts of the ribonuclease inhibitor in the cytoplasm. The inhibitor appears to be an almost ubiquitous feature in nature as in addition to mammalian, it has been reported in amphibian (Malicka-Blaskiewicz, 1978), avian (Kraus and Scholtissek, 1974) and insect (Aoki and Natori, 1981) tissues.

Preliminary studies were carried out on partially purified inhibitor from rat liver by Roth (1956, 1962) and Shortman (1961, 1962) who demonstrated that it was a heat- and acid- labile protein. The activity of the protein was dependent upon the integrity of one or more SH groups as it was inactivated by either sulphhydryl blocking reagents

e.g. p-hydromercuribenzoate (pHMB) or by heavy metals e.g. mercury and lead. In addition, inactivation of the inhibitor by sulphhydryl blocking reagents resulted in the release of active ribonuclease which had been bound to the inhibitor in a latent form. The partially purified inhibitor did not inhibit acid lysosomal ribonuclease but was specific for neutral ribonucleases of the pancreatic type.

#### **1.4.1 PURIFICATION OF THE RIBONUCLEASE INHIBITOR**

The ribonuclease inhibitor was initially purified from rat liver by a combination of ammonium sulphate precipitation of the high speed supernatant fraction of isotonic extracts of tissue, ion-exchange chromatography and gel filtration (Gribnau *et al.*, 1969). Purification to homogeneity was achieved with the introduction of an affinity chromatography step (RNase A coupled to CM-cellulose) as the final stage of purification (Gribnau *et al.*, 1970). This procedure was simplified by removing the ion-exchange chromatography and gel filtration steps which resulted in the rapid purification of an almost homogenous preparation of inhibitor (Gagnon and de Lamirande, 1973). Blackburn *et al.* (1977) obtained a homogenous preparation of inhibitor from the human placenta using ion-exchange chromatography and affinity chromatography on RNase A-Sepharose 4B. This purification procedure was simplified by utilising only ammonium sulphate precipitation and affinity chromatography (Blackburn, 1979) and high yields of human placental ribonuclease inhibitor were obtained (40-60%). Using a similar approach to that of Blackburn

(1979), the inhibitor has been purified to homogeneity from bovine brain (Burton, 1980), the livers of various mammalian species (Burton and Fucci, 1982) and rat testes (Fominaya *et al.*, 1988a).

The ribonuclease inhibitor is generally assayed by its ability to inhibit bovine pancreatic RNase A. One unit of activity is usually defined as the quantity required to inhibit the digestion of 5ng RNA with RNase A by 50%.

#### 1.4.2. GENERAL CHARACTERISTICS OF THE RIBONUCLEASE INHIBITOR

Using purified preparations of the inhibitor, further characterisation of the protein has been carried out and it appears that the ribonuclease inhibitor from different species and tissues show several common characteristics. The inhibitor is an acidic protein (Blackburn, 1977; Fominaya *et al.*, 1988a) with an isoionic point (pI) ranging from 4.6 for the human placenta to 5.1 for the rat testes. Optimal activity of the inhibitor occurs at pH7.5-8.0 and the activity is greatly decreased at pHs above 8.5. The relative molecular mass (Mr) of the inhibitor is circa 50,000 Daltons, as determined by both SDS polyacrylamide gel electrophoresis and gel filtration which indicates that it is a single polypeptide chain. When a complex of ribonuclease inhibitor and bovine pancreatic RNase A is subjected to gel filtration, the Mr of the complex is approximately 64,000 Daltons which suggests a 1:1 combination of the complex with RNase A on a molar ratio (Blackburn, 1977; Burton and Fucci, 1982). Native bovine brain ribonuclease complexed to its inhibitor gave a similar result (Burton *et al.*, 1980) thus indicating that the intracellular ribonuclease has a relative molecular mass similar to that of

RNase A (about 14,000 Daltons).

The inhibitor has also been detected in non-mammalian sources e.g. chick embryo cells (Kraus and Scholtissek, 1974), rooster liver (Dijkstra *et al.*, 1978), the insect *Ceratitis capitata* (Garcia-Segura *et al.*, 1985) and amphibian liver (Malicka-Blaskiewicz, 1978).

However, it has not been characterised as extensively as that from mammalian sources. All of the inhibitors from the non-mammalian sources appear to depend upon the maintenance of free sulphhydryl groups, a characteristic shared by their mammalian counterparts. The inhibitor from chicken liver has no inhibitory activity against mammalian ribonucleases but it does inhibit chicken type I ribonuclease (Dijkstra *et al.*, 1978) which suggests that the inhibitor may be species specific.

Studies have been carried out to determine the  $K_i$  value of the inhibitor and its mode of inhibition (Turner *et al.*, 1983; Fominaya *et al.*, 1988b and references therein). Turner utilised the methods of Dixon to determine the type of inhibition and also to determine the approximate  $K_i$  values for the interactions of porcine thyroid and liver inhibitors with RNase A. The Dixon plot showed competitive inhibition with  $K_i$  values of 0.4nM for liver and 0.1nM for thyroid. Analysis of the steady state velocities by the methods of Henderson and Bacci also showed that the porcine thyroid and liver ribonuclease inhibitors interact with RNase A by tight binding kinetics. In addition, Turner *et al.* showed that the inhibitor is of the slow binding type as the initial rate depended upon whether the enzyme or the substrate was used to start the reaction. More recently, Fominaya *et al.*, (1988b) described a general treatment which discriminates between the different types of inhibition and allows the calculation of



inhibition parameters, using ribonuclease inhibitor purified from rat testes as an example of a tight-binding inhibitor. They obtained a  $K_i$  value of  $3.22 \times 10^{-12} \text{M}$  which is several orders of magnitude lower than that obtained by Blackburn *et al.*, (1977), Burton *et al.*, (1980) and Turner *et al.*, (1983). The discrepancy in results between Turner *et al.* and Fominaya *et al.* (who both analysed the kinetics of ribonuclease inhibitor with procedures designed for tight binding inhibitors) may be explained as Turner *et al.* employed much higher concentrations of inhibitor and their protein preparations were impure. Thus, the  $K_i$  values for porcine inhibitor may be much lower than reported.

The inhibition of RNase A by the ribonuclease inhibitor has been well-studied (Blackburn *et al.*, 1977; Burton *et al.*, 1980) and the mechanism has been defined by Michaelis-Menten kinetics as pure, linear, non-competitive inhibition. However, inspection of the pertinent literature shows that the kinetic analyses of the ribonuclease:inhibitor system was carried out with an enzyme:inhibitor molar ratio of 1 i.e. the majority of the inhibitor molecules were present as a complex with RNase A. Also, the  $K_m$  values were several orders of magnitude greater than the  $K_i$  values and, under these conditions, Lineweaver-Burke plots invariably indicate non-competitive inhibition, although the inhibition may be otherwise. Further, studies carried out on the residues present at the active site of RNase A (Blackburn and Jailkhani, 1979; Blackburn and Gavilanes, 1980) showed that modification of only one amino acid, Lys-41, altered the binding of inhibitor to the enzyme and this was interpreted to support the idea of non-competitive inhibition. Turner *et al.*, (1983) proposed that this data was more suggestive of competitive inhibition as competitive inhibition involves the binding

of the inhibitor to the enzyme in such a way that catalysis is prevented through the active site being entirely or partially blocked. Similar kinetics result if binding of the inhibitor produces a conformational change which alters the positioning of the residues involved in either catalysis or binding of substrate. In addition, Turner states that if the inhibition of RNase A by the inhibitor is non competitive then, in theory, the inhibitor should be able to combine with both free enzyme and enzyme:substrate complex. In practice, the RNase A:inhibitor complex is readily available whereas a complex of RNase A:inhibitor:RNA has not been isolated.

Recently, Fominaya *et al.*, (1988b) suggested that to describe the regulation of inhibitory activity in terms of competition with cytoplasmic RNA for ribonuclease binding is not biologically meaningful. Instead they suggest that the activity of the inhibitor *in vivo* may be regulated through some modulation system which controls the reversibility of the inhibition equilibrium. They further propose that such a modulation system could be the GSSG/GSH (oxidised/reduced glutathione) which is known to be a cytoplasmic thiol buffer. This proposal is based upon the observation that all ribonuclease inhibitor proteins contain essential free thiol groups and all are inactivated by thiol blocking reagents.

#### 1.4.3 AMINO ACID ANALYSIS OF RIBONUCLEASE INHIBITOR

The amino acid composition has been determined from human placenta (Blackburn *et al.*, 1977), bovine brain (Burton *et al.*, 1980), the livers of five mammalian species (Burton and Fucci, 1982)

TABLE 1.1 AMINO ACID COMPOSITION OF MAMMALIAN  
RIBONUCLEASE INHIBITORS

This table illustrates the degree of conservation of amino acid composition among mammalian ribonuclease inhibitors. The data was obtained from the following sources:

1. Fominaya *et al.*, 1988a
2. Burton and Fucci, 1982
3. Burton *et al.*, 1980
4. Blackburn *et al.*, 1977

AMINO ACID	RESIDUES/MOLECULE			
	1 Rat testis	2 Rat liver	3 Bovine brain	4 Human placenta
Asx	55	59	43	47
Thr	25	23	20	16
Ser	42	43	40	45
Glx	61	60	65	64
Gly	40	33	53	36
Ala	30	29	38	34
Val	20	22	22	24
Met	2	2	2	2-3
Ile	13	10	9	12
Leu	66	89	88	85
Tyr	8	5	5	4
Phe	7	5	3	6
His	7	6	5	6
Lys	18	20	15	17
Arg	23	20	20	23
Cys	31	31	30	30
Trp	n.d.	5	5	5

and rat testes (Fominaya *et al.*, 1988a). As can be seen from Table 1.1, all ribonuclease inhibitors from these different sources have a very similar amino acid composition. Of special note is the high content of hydrophobic residues, especially leucine. It is also apparent that the inhibitor proteins contain a high level of cysteine and 1/2 cysteine residues (31 per molecule). Initially, it was calculated that 8 of these were present as free sulphydryl groups (Blackburn *et al.*, 1977). However, this figure has since been amended to approximately 25 (Blackburn, personal communication).

The data obtained from the amino acid composition suggests that there has been a degree of evolutionary conservation, at least in mammals. The data also predicts that the primary sequence of the inhibitors would be homologous. However, Burton and Fucci (1982) raised antiserum against human placental ribonuclease inhibitor and, using this antiserum, showed that the immunological cross reactivity between human placental inhibitor and a variety of mammalian liver inhibitors was relatively low. This suggests that either there may be significant variations in the primary structure of ribonuclease inhibitors from different species or the human placental inhibitor differs from the liver inhibitors.

Very recently, and towards the end of the study described in the results section of this thesis, the nucleotide sequence of human ribonuclease inhibitor (Schneider *et al.*, 1988; Lee *et al.*, 1988) and the amino acid sequence of inhibitor from both human placenta and porcine liver became available (Lee *et al.*, 1988; Hofsteenge *et al.*, 1988). Hofsteenge *et al.*, (1988) determined the amino acid sequence of ribonuclease inhibitor purified from porcine liver. Digestion of the protein with cyanogen bromide resulted in the isolation of three peptides (CB1, CB2 and CB3). Sequence analysis of CB1 revealed

that the N terminus of the inhibitor is blocked with an acetyl group. Overlapping sequence was obtained by digesting each of the peptides with either trypsin or V8 protease from *S. aureus* and also by digesting the whole protein with the same enzymes. Thus, the total amino acid sequence was obtained.

The primary amino acid sequence of porcine liver ribonuclease inhibitor revealed that the protein exhibits a considerable amount of internal homology. Two different types of internal repeat were apparent which occurred alternatively. The type A repeat was 29 amino acids long whereas the type B repeat was 28 amino acids. Both repeats were rich in leucine residues which were present at constant positions.

The amino acid sequence of inhibitor from human placenta has also been determined (Lee *et al.*, 1988) by sequencing of tryptic digests of the protein. The ribonuclease inhibitor from human placenta has a stretch of 5 amino acids more at the N terminus than the protein isolated from pig. The placental inhibitor also has a blocked N terminal and has seven direct internal repeats, each 57 amino acids in length.

The primary structure of ribonuclease inhibitor is similar to six other proteins of diverse function which contain leucine-rich repeats: a subunit of human platelet glycoprotein 1b (Titani *et al.*, 1987; Lopez *et al.*, 1987); human leucine-rich  $\alpha_2$  glycoprotein of unknown function (Takahashi *et al.*, 1985); human fibroblast proteoglycan core protein (Krusius and Ruoslahti, 1986; Patthy, 1987); yeast adenylate cyclase, a membrane-bound enzyme that converts ATP into cAMP (Kataoka *et al.*, 1985); *Drosophila Toll* protein (Hashimoto *et al.*, 1988) and *Drosophila* chaoptin (Reinke *et al.*, 1988). The

ribonuclease inhibitor appears to be unique as it is the first cytoplasmic protein with such a repeat. It has been suggested that this leucine-rich repeat is important in membrane binding (Takahashi *et al.*, 1985; Hashimoto *et al.*, 1988). However, early studies on the inhibitor (Roth, 1956) have shown that little inhibitor activity is associated with membrane fractions. Thus, the leucine-rich repeats may form structures that have other functions e.g. protein-protein interactions. Adenylate cyclase, glycoprotein 1b and inhibitor are all known to bind to other proteins.

It is interesting to note that ribonuclease inhibitor binds with a 1:1 stoichiometry which suggests that the binding site for ribonuclease does not correspond to a single leucine-rich repeat but is formed by a structure resulting from an interaction of the repeats in the protein. Other proteinase/proteinase inhibitor interactions show a relationship between the stoichiometry and the number of domains e.g. adzuki bean inhibitor I binds to two molecules of trypsin and the inhibitor has two homologous repeated domains (Yoshida and Yoshikawa, 1975; Kiyohara *et al.*, 1981).

#### **1.4.4 INTERACTION OF THE INHIBITOR WITH RNASE A**

The interaction of the inhibitor with RNase A has been well-studied. Specific proteolytic cleavage of RNase A, which removed parts of the active site, was performed and the resultant derivatives assayed for inhibitor binding by competition binding assays (Blackburn and Jailkhani, 1979). The results demonstrated that the active site residues, His-112 and His-119, and the auxiliary residues Lys-7, Phe-120, Asp-121 and Ser-123 are not essential for the

**TABLE 1.2 CONTACT POINTS OF RNASE A WITH**  
**RIBONUCLEASE INHIBITOR**

The three regions on RNase A which are thought to interact with the inhibitor are shown. The data was obtained from Blackburn and Gavilanes, 1982.



**Group A**      Lys-7, Lys-41, Pro-42, Val-43, Lys-91, Tyr-92 and  
Pro-93

**Group B**      Lys-31 and Lys-37

**Group C**      Lys-61 and surrounding residues

interaction of RNase A with the inhibitor. Also, the removal of the residues 1 to 20 inclusive did not affect the binding with inhibitor.

Specific chemical modifications of amino acid side chains on the enzyme showed that four arginine residues are not involved in binding RNase A to the inhibitor (Blackburn and Gavilanes, 1980). However, modifications made to lysines indicated that these residues are important for the interaction. When the inhibitor is bound to RNase A, the enzyme is protected from inactivation by reagents which are known to inactivate RNase A by reaction at Lys-41. Moreover, specific modifications of Lys-41 decreased the interaction with the inhibitor by 90%. Thus this residue appears to be involved in the interaction with the inhibitor.

The ability of the chemically-modified derivatives to bind placental inhibitor was also monitored by circular dichroism (CD). The CD measurements suggested that at least one tyrosine residue of RNase A is involved in binding the enzyme to the inhibitor. A comparison of the ability of several different mammalian pancreatic ribonucleases to bind human placental inhibitor was carried out to determine which tyrosine residue(s) was involved. The binding of the different ribonucleases with inhibitor were very similar and, as Tyr-92 is the only tyrosine residue to be retained in all of the species studied, it was concluded that this residue may play an important role in the interaction. Carboxymethylation of the active site residue His-119 increases the binding of enzyme to inhibitor (Blackburn and Gavilanes, 1980) and it is thought that this modification renders Tyr-92 more accessible for interaction of RNase A with inhibitor.

From X-ray analysis of RNase A and from the above data, the contact points with the inhibitor can be placed into 3 groups (Table 1.2). Groups A and B are adjacent, whereas group C lies distal to

both groups. It is proposed that there is extensive contact between the inhibitor and RNase A with the inhibitor molecule wrapping around the RNase A molecule, leaving the active site cleft exposed.

As mentioned in the previous section, amino acid analyses has shown that there are 31 cysteine and 1/2 cysteine residues per molecule of ribonuclease inhibitor. Addition of reagents which react with sulphhydryl groups results in both inactivation of inhibitor and dissociation of the ribonuclease:inhibitor complex. These observations suggest that free sulphhydryl groups may play an essential role in the binding of inhibitor to ribonuclease. Work carried out by Blackburn (personal communication) showed that of the 25 free sulphhydryl groups of human placental inhibitor, at least 12 are exposed to the solvent and react readily with alkylating reagents. Of 4-6 of the most rapidly reacting sulphhydryl groups, 2 appear to be most important for full inhibitor activity.

#### **1.4.5 FUNCTION OF THE RIBONUCLEASE INHIBITOR**

It is clear that cellular ribonucleases cannot be catalytically unrestrained or all rRNA, tRNA and mRNA would be subject to uncontrolled degradation. The two known classes of mammalian cellular ribonucleases (described in Section 1.3) are kept under control either by compartmentation, as with the localisation of type II enzymes in lysosomes, or by the ribonuclease inhibitor protein. However, this raises the question what controls the release of type I ribonuclease from its inhibitor or for that matter the entry of RNA and/or RNA-containing subcellular particles into lysosomes? There are two main possibilities neither of which are mutually exclusive. On the

one hand, changes to the structure, compartmentation or macromolecular associations of RNA molecules could make them more vulnerable to low levels of unrestrained enzyme. Alternatively, the levels of available enzyme could be controlled for instance by changes in the relative levels of the enzyme and its inhibitor.

There are several lines of evidence which suggest that the interaction between ribonuclease inhibitor and ribonuclease may play an important role in the control of RNA metabolism and the regulation of cell growth and differentiation. In general, tissues which are characterised by high rates of RNA synthesis and accumulation have increased ratios of inhibitor to ribonuclease activities i.e. there is an excess of unbound inhibitor in these systems. The ribonuclease inhibitor:ribonuclease ratio is increased in regenerating rat liver (Moriyama *et al.*, 1969), in thyroid-stimulating hormone (TSH) treated rat thyroid relative to normal glands (Grief and Eich, 1972), in developing and lactating rat mammary gland (Liu *et al.*, 1975), in oestrogen-stimulated rooster liver (Dijkstra *et al.*, 1978) and in mitogen-stimulated lymphocytes (Kyner *et al.*, 1979).

The aforementioned tissues i.e. liver, thyroid and mammary gland are associated with the synthesis of major abundant proteins e.g. albumin and other serum proteins are synthesised in the liver and the major milk proteins are synthesised in the lactating mammary gland. Other examples of tissues involved in synthesising major abundant proteins are reticulocytes (Aoki *et al.*, 1981) where globin proteins are synthesised, calf lens (Ortworth and Byrnes, 1971) where alpha-crystallin is synthesised and skeletal muscle (Little and Whittingham, 1981) where the major muscle proteins are synthesised. All of these tissues have been shown to contain high levels of unbound inhibitor and this is further evidence that the ratio of inhibitor to ribonuclease

may be implicated in the metabolism of RNA.

Conversely, tissues with decreased levels of protein synthesis and increased catabolic activity demonstrate lower inhibitor and elevated ribonuclease activities. Examples of this phenomena include the muscle of dystrophic mice compared to normal mice (Little and Meyer, 1970), the liver of rats after adrenalectomy (Lui and Matrisian, 1977) and several tissues of the rat during aging (Chesters and Will, 1978). Tissues which are not associated with the synthesis of major abundant proteins e.g. lung, spleen and kidney have low levels of ribonuclease activity (Brockdorff, PhD thesis, 1985).

Studies carried out by Sajdel-Sulkowska and Marotta (1984) suggested that there is an increase in free alkaline ribonuclease activity and a corresponding decrease in the level of inhibitor in the brains of Alzheimer's-diseased (AD) patients relative to normal brain. An increase in ribonuclease activity may result in an increase in the rate of degradation of RNA which would lead to a decrease in protein synthesis, a known symptom of Alzheimer's disease. However, Jones and Knowler (1989) measured the activity of total and free ribonuclease and ribonuclease inhibitor from various pathologically affected and unaffected regions of both AD brain and age-matched controls and demonstrated that there are no observable differences in the levels of ribonuclease and inhibitor between the brains of patients suffering from AD and control brains. The results of Jones and Knowler are in agreement with those of two other groups who were also unable to demonstrate any differences in ribonuclease levels in AD and normal brains (Guillemette *et al.*, 1986; Morrison *et al.*, 1987)

There are deviations from the general rule that increased protein

synthesis results in an increased ratio of inhibitor to ribonuclease e.g. hepatomas of the rat liver. In general, hepatomas are characterised by increased growth and cellular proliferation and it would be expected that this may result in an increased ratio of inhibitor to ribonuclease. Indeed the level of free inhibitor in Dunning hepatoma and the Morris 7288C hepatoma is higher than that found in normal rat liver (Roth, 1967). However, the level of free inhibitor in Novikoff hepatoma (Gauvreau *et al.*, 1974) and the Morris 4123D hepatoma (Roth, 1967) is reduced compared to normal liver. Thus, there does not appear to be a clear cut relationship between the level of free inhibitor and increased growth in rat liver hepatomas.

Another exception to the rule that tissues with increased levels of protein synthesis have a high ratio of inhibitor to ribonuclease is the rat uterus. In the uterus of the immature rat, the level of free inhibitor is similar to that in rat liver i.e. the inhibitor is present in excess over ribonuclease. Treatment with oestrogen (McGregor *et al.*, 1981; Brockdorff and Knowler, 1986) or during the course of normal development (Munro and Knowler, 1982) results in the loss of inhibitor activity. These effects are due to the action of oestrogen as ovariectomy of mature animals results in the partial restoration of inhibitor activity (Zan-Kowalszewska and Roth, 1975). The mechanism by which oestrogen decreases ribonuclease inhibitor activity was determined by Brockdorff and Knowler (1986) by raising anti-serum to purified rat liver ribonuclease inhibitor to quantitate the total levels of this protein in uterine preparations. They found that 4 days after treatment with oestrogen the total level of inhibitor had increased approximately 1.5 fold whereas cytoplasmic ribonucleases increased approximately 7 fold. Thus the free inhibitor becomes saturated with ribonuclease and inhibitor activity is observed to

decrease.

At present, the reasons for these deviations from the general rule are not fully understood. The hormone-stimulated uterus and hepatoma cells are undergoing rapid growth and cell differentiation. In this situation, increased ribonuclease activity may be required to degrade the mRNA of transiently expressed proteins which are involved in the cell cycle. The other systems in which ribonuclease has been studied have been fully differentiated tissues e.g. brain, kidney and liver. As mentioned, these tissues are involved in the synthesis of major abundant proteins and the mRNA encoding these proteins has been found to be relatively stable and therefore ribonuclease activity may not be required. Kraft and Shortman (1970 ) suggested that the heterogeneity of cell types within a given tissue may account for the deviations from the general rule. This is exemplified by the rat kidney (Lui and Matrisian, 1977) where there is no observable inhibitor activity in total kidney or kidney cortex preparations. However, free inhibitor is observed in red or white medulla preparations.

Further evidence that the inhibitor plays an essential role in the control of RNA metabolism and protein synthesis includes the observation that ribonuclease inhibitor stabilises mRNA in cell-free translation systems, thus increasing the size of the polypeptides synthesised (Robbi and Lazarow, 1978; Scheele and Blackburn, 1979). Also, addition of ribonuclease inhibitor to *in vitro* transcription systems improves the synthesis of RNA (Eichler *et al.*, 1981) and improves the yield of high molecular weight cDNA obtained in reverse transcription systems (de Martinoff, 1980). Moreover, polysome stability is enhanced in the presence of inhibitor. Isolation

of intact polysomes is easier from tissues with high endogenous levels of inhibitor (Blobel and Potter, 1966; Takahashi *et al.*, 1966) and can be improved in many tissues by exogenous addition of inhibitor (Burghouts *et al.*, 1970).

As mentioned in Section 1.4.2, it has been proposed that the ribonuclease:ribonuclease inhibitor system may be regulated through a modulation system *in vivo* (Fominaya *et al.*, 1988b) which may control the reversibility of the inhibition equilibrium. The authors propose that the GSSG/GSH system (oxidised/reduced glutathione), a known cytosolic thiol group buffer, may be involved in the regulation of the system. It has been shown that oxidised glutathione reversibly inactivates the inhibitor from human placenta (Blackburn, personal communication). A relationship between inhibitor and oxidised glutathione has been implied *in vivo* as calf lens cataractogenesis is accompanied by decreased inhibitor and increased ribonuclease activity (Maione *et al.*, 1968), together with increased levels of oxidised glutathione (Reddy *et al.*, 1976). As yet, there is no evidence that a similar system is operative in other tissues.

Ribonuclease and ribonuclease inhibitor activities are associated with free mRNP particles of the human placenta (Gileadi *et al.*, 1984) and it is possible that a function of the complex may be to control the expression and turnover of mRNPs. However, less than 1% of the total inhibitor of placental cytoplasm is associated with mRNP particles a finding which raises some doubt about the significance of this data. At present, it is unknown if the ribonuclease:inhibitor complex is also associated with polyribosome mRNPs.

An alternative hypothesis as to the function of the ribonuclease inhibitor is that it possibly acts as a scavenger protein, inhibiting type I ribonucleases that may be taken up into cells from serum or released



from intracellular organelles. This hypothesis results from the finding that there are a number of endo- and exoribonucleases which occur in the ribosomal fraction of eukaryotic cells which are thought to be involved in the degradation of mRNA in eukaryotes but which are insensitive to the inhibitor (Reboud *et al.*, 1976; Kumagai *et al.*, 1985a,b).

#### 1.4.6 ASSOCIATION OF RIBONUCLEASE INHIBITOR WITH ANGIOGENIN

Recently it has been suggested that, in addition to controlling cytoplasmic ribonucleases, the inhibitor may also be involved in the regulation of angiogenesis (Shapiro and Vallee, 1987). Angiogenin is a protein that induces blood vessel growth (Fett *et al.*, 1985) and has been isolated from tumour cell conditioned medium and from human plasma (Fett *et al.*, 1985; Shapiro *et al.*, 1987). The primary structure of angiogenin is approximately 35% identical with human pancreatic ribonuclease (Strydom *et al.*, 1985). Angiogenin exhibits relatively low ribonucleolytic activity, with a specificity different from that of pancreatic ribonuclease. Angiogenin catalyses the limited cleavage of 28S and 18S rRNA into products of 100-500 nucleotides long and its primary function is probably not as a ribonuclease. Also, the role of angiogenin as an angiogenic factor has also been questioned (Marx, 1987).

On the basis of angiogenin's structural and catalytic similarity with pancreatic ribonucleases, the interaction of angiogenin with human placental ribonuclease inhibitor was investigated. Angiogenin was found to bind tightly to the inhibitor and this results in the

inhibition of both its angiogenic and ribonucleolytic activities (Shapiro and Vallee, 1987). Angiogenin and placental ribonuclease inhibitor form a very tight 1:1 complex and the  $K_i$  value has been calculated to be  $7.1 \times 10^{-16} \text{M}$ , which is approximately 60 times lower than that for bovine pancreatic RNase A (Lee *et al.*, 1989). As with bovine pancreatic RNase A, the inhibition of angiogenin is reversible as addition of pHMB dissociates the complex to yield active angiogenin.

As described in Section 1.4.4, Blackburn and coworkers suggested that there are 3 contact regions for ribonuclease inhibitor in the 3-D structure of RNase A: 1) Lys-7, Lys-41, Pro-42, Val-43, Lys-93, Tyr-92 and Pro-93; 2) Lys-31 and Lys-37 and 3) Lys-61 and adjacent residues. In these regions RNase A and angiogenin are very similar and therefore it may be expected that both enzymes will bind placental inhibitor with comparable avidity. As many of these residues are outwith the active centre, the conservation of residues necessary for enzymatic activity alone cannot account for the strength of the interaction. This implies that the capacity of angiogenin to bind placental inhibitor has been maintained independently during evolution. Notwithstanding doubts about the true function of angiogenin, it would therefore seem plausible that binding of inhibitor reflects a physiologically relevant control mechanism.

**CHAPTER TWO**

**MATERIALS AND METHODS**

## 2.1 LIST OF SUPPLIERS

All chemicals used were of analytical grade and were obtained from BDH Chemicals Limited , Formachem (Research International) Limited or Fisons Scientific Apparatus. Where chemicals and equipment were obtained from other sources, this is indicated in the text. A list of the suppliers is given below.

Amersham International plc, White Lion Road, Amersham, Bucks HP7  
9LL

BDH Chemicals Limited, Poole, Dorset, England.

Boehringer-Mannheim, BCL, Boehringer Mannheim House, Bell Lane,  
Lewes, East Sussex.

James Burrough Limited, 70 Eastways Industrial Park, Witham, Essex.

Cambridge Biotechnology Labs., 12-14 St. Ann's Crescent, London  
SW18

Difco Laboratories, Detroit, Michigan, U.S.A.

Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

Fluka Fluorochem Ltd., Peakdale Road, Glossop, Derbyshire SK13  
9XE

Formachem. Ltd., Strathaven, Scotland, U.K.

FSA Lab Supplies, Bishop Meadow Road, Loughbough, Leics., LE11 0RG

GIBCO-BRL Limited, P.O. Box 35, Paisley, Scotland.

Genetic Research Instrumentation Ltd., Gene House, Dunmow Road,  
Felsted, Dunmow, Essex CM6 3LD

May and Baker Limited, Dagenham, England.

Pharmacia LKB Biotechnology, Pharmacia House, Midsummer  
Boulevard, Milton Keynes, MK9 3HP

Schleicher and Schuell, Dassel, W. Germany.

Scottish Antibody Production unit, Law Hospital, Carlisle,  
Lanarkshire, Scotland.

Siemens Ltd., 26-28 Napier Court, Wardpark North, Cumbernauld.

Sigma Chemical Company, Fancy Road, Dorset, England BH17 7NH.

Technical Photo Systems Ltd., 34 Telford Road, Lenziemill Industrial  
Estate, Cumbernauld G67 2AX

Whatman Ltd., Maidstone, Kent, U.K.

## 2.2 STERILE PRECAUTIONS

To prevent contamination of glasswear and solutions by ribonucleases, the following precautions were taken:

- (i) disposable protective gloves were worn during all procedures;
- (ii) all solutions and glassware were autoclaved at 15 p.s.i. for 20 minutes;
- (iii) disposable plasticware was treated with Repelcote (dimethyldichlorosilane in 1,1,1-dichloroethane, BDH) before being autoclaved as described above.

## 2.3 EXPERIMENTAL ANIMALS

All animals were supplied by the departmental animal house. The animals were given free access to food and water.

The adult rats used were of the Wistar strain and weighed approximately 250g. These animals were killed by concussion and cervical dislocation.

The rabbits used for the production of antiserum were of the White New Zealand breed.

## 2.4 GENERAL PROTEIN METHODS

### 2.4.1 Protein Assays

In general, protein concentrations were measured using the dye-binding method of Bradford (1976) as modified by Macart and Gerbaut (1982).

The Bradford dye reagent was prepared as follows:

100mg Coomassie Brilliant Blue G (Sigma) and 30mg SDS were dissolved in 50ml 95% (v/v) ethanol and then 100ml 85% (v/v) phosphoric acid was added. The solution was diluted to a final volume of 1000ml and filtered to remove particulate matter. This reagent was found to be stable for several months when stored at 4°C in the dark.

1ml of the dye reagent was added to 100µl of each sample to be assayed. After 15 minutes, the absorbance at 595nm was measured against a reagent blank prepared from 100µl of the appropriate buffer and 1ml of dye reagent. Standard curves were prepared using various amounts of a 1mg/ml solution of BSA (Sigma).

The protein concentration of fractions from chromatographic columns was estimated by measuring the absorbance at 280nm. One absorbance unit at 280nm was taken to be equivalent to approximately 1mg/ml protein.

A Cecil 272 spectrophotometer was used to measure absorbance at 280nm and 595nm.

#### 2.4.2 Concentration of Protein Samples

Proteins were concentrated by two methods, depending upon the initial volume of the protein solution to be concentrated.

For samples of 1ml or less, the protein was precipitated by the addition of 4 volumes of acetone at -20°C overnight. The proteins were recovered by centrifugation at 3,000 rpm (1,500xg<sub>av</sub>) in a Beckman benchtop centrifuge for 10 minutes. Residual acetone was evaporated in a stream of N<sub>2</sub> gas.

Samples of proteins in larger volumes were concentrated by lyophilisation in a Flexi-Dry freeze dryer attached to a vacuum pump.

### 2.4.3 Polyacrylamide Gel Electrophoresis

Electrophoresis in the presence of 0.1% (w/v) SDS was carried out using the discontinuous system described by Laemmli (1970), utilising the procedure of Le Sturgeon and Beyer (1977).

The following stock solutions were prepared:

- A. 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide;
- B. 1.5M Tris.HCl, pH8.8;
- C. 1.5M Tris.HCl, pH6.8;
- D. 10% (w/v) SDS;

Reservoir Buffer: 0.025M Tris, 0.192M glycine, 0.1% (w/v) SDS. The approximate pH of this buffer was 8.3.

The percentage of the resolving gel varied from 7.5% to 15% depending upon the molecular weight of the proteins under analysis. In all cases, a 3% stacking gel was used. The gel mixtures were prepared using the volumes indicated in Table 2.1. In each case, the gel mixture, except for TEMED (NNN'N'-tetramethylethylenediamine, BDH), was prepared in a Buchner flask and degassed for 2 minutes using a water suction pump. Polymerisation was initiated by the addition of TEMED to the gel mixture. The resultant slab gels (15x18cm, 1mm thick) were electrophoresed using a BRL V161 vertical gel electrophoresis system at a constant current of 40-50mA until the tracking dye reached the bottom of the gel.

### 2.4.4 Preparation of protein samples for electrophoresis

Samples to be resolved by SDS polyacrylamide gel electrophoresis



**TABLE 2.1 COMPOSITION OF SDS POLYACRYLAMIDE GELS**

This table shows the composition of SDS polyacrylamide gels used as outlined in Section 2.4.3. The stock solutions A,B,C and D are described in the text of the aforementioned section.

SOLUTION	RESOLVING GEL			STACKING GEL
	(% (w/v) polyacrylamide)			
	7.5%	10%	15%	3%
A (ml)	10	13	20	2
B (ml)	10	10	10	-
C (ml)	-	-	-	5
D (ml)	0.4	0.4	0.4	0.2
Water	19.6	15.6	9.6	12.8
Ammonium persulphate (mg)	30	40	50	20
TEMED ( $\mu$ l)	20	20	20	10

were diluted with an equal volume of Laemmli sample buffer (0.0725M Tris.HCl pH6.8, 3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v)  $\beta$ -mercaptoethanol, 0.002% (w/v) bromophenol blue, (Sigma), then boiled for 2 minutes at 100°C.

#### 2.4.5 Staining of polyacrylamide gels

##### A. Staining with Coomassie Brilliant Blue R

Gels were stained for protein in 0.25% (w/v) Coomassie Brilliant Blue R (Sigma), 25% (v/v) methanol, 10% (v/v) acetic acid at room temperature for 2 hours. Occasionally, if a gel had to be stained quickly, the gel was placed in stain at 37°C for 30 minutes. The background was destained by diffusion in 50% (v/v) methanol, 10% (v/v) acetic acid for several hours at room temperature with several changes of buffer.

##### B. Staining with silver.

When greater sensitivity was required, proteins were stained with silver using the method of Wray *et al.*, 1981.

Gels were soaked overnight at room temperature in 50% (v/v) methanol. Staining solution was prepared by adding solution A (0.8g silver nitrate in 4ml H<sub>2</sub>O) to solution B (1.4ml 14.8M ammonia plus 21ml 0.36% (w/v) NaOH) dropwise and stirring vigorously. The solution was then made to 100ml with double deionised distilled water. The gel was incubated at room temperature in staining solution for 8 minutes, then rinsed for one hour with 6 changes of water. The stain was developed by immersing the gel in a solution comprising 2.5ml 1%

(w/v) citric acid and 0.25 ml 38% (v/v) formaldehyde in 500ml water.  
Staining was stopped by washing the gel in water

#### 2.4.6 Preparative SDS gel electrophoresis

Preparative slab gels were prepared and run exactly as described in Section 2.4.3, except that 3mm spacers were used and a single continuous well was formed in the stacking gel for the application of sample.

#### 2.4.7 Peptide mapping

Peptide mapping was performed on fusion protein and ribonuclease inhibitor essentially as described by Cleveland *et al.* (1977)

Crude cell lysate (Section 2.11.2) containing induced fusion protein was electrophoresed on a 7.5% SDS polyacrylamide preparative gel (Section 2.4.6). The gel was stained and destained as described in Section 2.4.5.A. The band corresponding to the fusion protein was excised and cut into strips of gel of approximately 5mm. Gel slices containing ribonuclease inhibitor were obtained in a similar fashion except that the inhibitor was electrophoresed on a 10% SDS polyacrylamide preparative gel. The gel slices were soaked for 30 minutes in a solution containing 0.125M Tris.HCl pH6.8, 0.1% (w/v) SDS, 1mM EDTA. The slices could be stored in this buffer at -20°C until required.

A 15% SDS polyacrylamide gel was prepared as described in Section 2.4.3 with the additional inclusion of 1mM EDTA. The gel was cast with a longer than usual stacking gel (approximately 1cm longer) and a

wider-toothed comb was used.

The gel slices, prepared as above, were pushed to the bottom of a well which had been filled with the above buffer. Any spaces around the gel were filled by overlaying the gel with the same buffer made 20% (v/v) with glycerol. Finally, 10 $\mu$ l of buffer containing 10% (v/v) glycerol and an amount of protease that varied experimentally was overlaid into each well. Electrophoresis was performed in the usual manner except that the current was switched off for 1 hour when the tracking dye neared the bottom of the stacking gel.

After electrophoresis, the resolved peptides were transferred to nitrocellulose membrane and analysed by western blotting as described in Section 2.7.3.

#### 2.4.8 Determination of the relative molecular weight of a protein by SDS PAGE.

Relative molecular mass (Mr) values were obtained from SDS polyacrylamide gels after staining for protein by determining the electrophoretic mobility (Rf) values as follows:

$$R_f = \frac{\text{distance migrated by protein}}{\text{distance migrated by tracker dye}}$$

A plot of Rf values versus log Mr of a set of standard proteins enabled the Mr of the protein under analysis to be determined.

The standard proteins were obtained from Sigma and consisted of myosin (200,000 Da), phosphorylase B (97,000 Da), albumin (68,000 Da), ovalbumin (45,000 Da) and carbonic anhydrase (30,000 Da).

## 2.5 PURIFICATION OF RIBONUCLEASE INHIBITOR FROM RAT LIVER

### 2.5.1 Preparation of the RNase A-Sepharose 4B affinity column

The affinity column was prepared by the method outlined in "Principles of affinity chromatography" (Pharmacia).

1.5g of freeze-dried CNBr-activated Sepharose 4B (Sigma) was swollen in 300ml 1mM HCl for 15 minutes. The acid solution was removed using a sintered glass filter (porosity G3) fitted to a vacuum pump. The gel slurry was washed with 10ml coupling buffer (0.1M sodium bicarbonate pH8.3, 0.5M NaCl) and immediately transferred to a solution of the ligand i.e. 10mg bovine pancreatic RNase A (Sigma) in 5ml coupling buffer. The suspension was then mixed on an end-to-end rotating wheel for either 2 hours at room temperature or for 16 hours at 4°C. After the coupling reaction, any remaining active groups were blocked by mixing with blocking buffer (0.2M glycine pH8) at room temperature for two hours. Excess adsorbed protein was removed by washing with 200ml coupling buffer, then with 200ml wash buffer (0.1M acetate buffer pH4, 0.5M NaCl ) and then finally with 200ml coupling buffer.

1ml of the gel slurry was poured into the barrel of a 2ml sterile disposable syringe which had been stoppered with glass wool and a nitrocellulose (Schleicher and Schuell) filter. The column was then equilibrated with 0.045M potassium phosphate pH6.4, 0.01M  $\beta$ -mercaptoethanol, 0.001M EDTA at 4°C.

### 2.5.2 Purification procedure

Ribonuclease inhibitor was purified from rat liver using the affinity chromatography procedure of Burton *et al.* (1980). All steps were carried out at 4°C, unless otherwise stated.

Livers from 20 male Wistar rats were excised and homogenised in 3 volumes of ice-cold homogenisation buffer (0.05M Tris.HCl pH7.6, 0.35M sucrose, 0.025M KCl, 0.01M Mg(CH<sub>3</sub>COO)<sub>2</sub>, 0.01M β-mercaptoethanol) in an UltraTurrax homogeniser. The homogenate was then centrifuged for 2 hours at 25,000 rpm (105,000xg<sub>av</sub>) in the SW.27 swing-out rotor of a Beckman L5-50 ultracentrifuge

The supernatant fraction obtained from the high speed spin was brought to 35% (w/v) saturation with the gradual addition of ammonium sulphate (free from heavy metals), stirred for 30 minutes and centrifuged at 12,000 rpm (25,000xg<sub>av</sub>) in the GSA rotor of a Sorvall RC-5 centrifuge for 15 minutes. The supernatant fraction was brought to 60% (w/v) ammonium sulphate saturation, then stirred and centrifuged as described above. The resultant pellets were used immediately or stored at -20°C until required.

The pellets were resuspended in a minimal volume of starting buffer (0.045M potassium phosphate pH6.4, 0.01M β-mercaptoethanol, 0.001M EDTA ) and dialysed against 2x20 volumes of the same buffer for 4 hours. The dialysate was centrifuged at 17,000 rpm (70,000xg<sub>av</sub>) in a Beckman SW.27 rotor for one hour to remove particulate matter. The supernatant fraction was then loaded onto a RNase A-Sepharose affinity column (Section 2.5.1) at a flow rate of 25ml/hour. The run-through fraction containing the proteins that had not bound to the column was retained for use at a later stage (see Section 2.7.5). The column was washed with starting buffer made 0.5M with NaCl until all

non-specifically bound proteins were eluted. Ribonuclease inhibitor was then eluted with elution buffer (0.05M borate buffer pH6, 4M NaCl, 0.01M  $\beta$ -mercaptoethanol 0.001M EDTA, 15% (v/v) glycerol) at a flow rate of 10ml/hour. All fractions were assayed for ribonuclease inhibitor (Section 2.5.4) and for protein by measuring absorbance at 280nm. Fractions containing inhibitor were pooled and dialysed against 2x20 volumes of dialysis buffer (0.02M Tris.HCl pH 7.5, 0.15M NaCl, 0.001M EDTA, 0.01M  $\beta$ -mercaptoethanol, 15% (v/v) glycerol) for 4 hours, then stored at -20°C.

### 2.5.3 FPLC fractionation of ribonuclease inhibitor

When required, ribonuclease inhibitor was further purified by ion-exchange chromatography on the FPLC system (Pharmacia) using the Mono Q anion-exchange column. Fractions from the affinity chromatography column which contained ribonuclease inhibitor were dialysed for 16-20 hours at 4°C against 100mM Tris.HCl pH7.4, 10mM  $\beta$ -mercaptoethanol, 1mM EDTA, with several changes of buffer. The Mono Q column was equilibrated with the same buffer and the protein loaded onto the column. Ribonuclease inhibitor was eluted with a 0-0.5M linear NaCl gradient over a period of 30 minutes at a flow rate of 1ml/minute and 1ml fractions were collected.

### 2.5.4 Assay of ribonuclease inhibitor activity

The assay used to detect ribonuclease inhibitor activity was described by Brockdorff and Knowler (1986) and is based upon the ability of the ribonuclease inhibitor to prevent the digestion of



transfer RNA by RNase A.

Samples were incubated at 37°C for 30 minutes in a total assay volume of 150µl consisting of 0.025% (w/v) BSA (fraction V, Sigma), 0.25ng bovine pancreatic RNase A (Sigma) and 125µg tRNA (Sigma) in 100mM Tris.HCl pH7.5 with 1mM EDTA. The reaction was initiated by the addition of tRNA to the samples and stopped by the addition of 50µl ice-cold 24% (v/v) perchloric acid in 40mM lanthanum acetate (Ventron). Under these conditions, the digestion of tRNA by RNase A was linear. The samples were incubated on ice for 20 minutes and centrifuged in an Anderman 5415 microfuge for 5 minutes. 100µl of supernatant fraction, containing acid-soluble nucleotides, was diluted to 1ml with water and the absorbance read at 260nm on a Cecil CE 272 spectrophotometer. The assay was carried out in sterile 1.5ml polypropylene microtubes (Eppendorf tubes) which had been treated with Repelcote (dimethyldichlorosilane in 1,1,1-dichloroethane)

One unit of inhibitor activity is defined as the amount required to inhibit by 50% the degradation of RNA by 5ng of RNase A. As this assay uses 0.25ng RNase A, the quantity of inhibitor resulting in 50% inhibition was taken to be 0.05 units.

## **2.6 PREPARATION OF PEPTIDES FOR AMINO ACID SEQUENCING**

### **2.6.1 Preparation of ribonuclease inhibitor for digestion by proteases.**

In general, proteins in their native state are digested only to a limited extent by proteases. This is probably due to the conformation of the

protein which renders susceptible peptide bonds inaccessible to the protease. Also, the presence of covalent intrapolypeptide crosslinks e.g. disulphide bonds may obstruct the action of the protease. Thus, to render the protein more accessible to the action of proteases, the protein must be fully denatured, the disulphide bonds cleaved and the resultant sulphhydryl groups alkylated.

A preparation of FPLC-purified ribonuclease inhibitor (Section 2.5.3) was dialysed against distilled water for 48 hours at 4°C with several changes of water. The sample was then transferred to an acid-washed round bottom flask and lyophilised overnight. The precipitate was resuspended in 4ml de-gassed 6M guanidine hydrochloride (Sigma) in 100mM Tris.HCl pH8.5. A five times molar excess of dithiothreitol (DTT) over protein cysteine residues was added and the solution stirred under N<sub>2</sub> for 3 hours at room temperature. A five times molar excess of iodoacetic acid (IAA) over DTT was then added and the solution stirred in the dark for 3 hours over N<sub>2</sub> at room temperature. This alkylating reaction was quenched with ten times molar excess β-mercaptoethanol over IAA. The protein was then dialysed against distilled water for a further 3 days and then lyophilised overnight

#### 2.6.2 Proteolytic digestion of ribonuclease inhibitor

The lyophilised protein, obtained as described in Section 2.6.1, was resuspended in 4ml 50mM ammonium bicarbonate pH8.5. The sample was then divided into two: one half of the sample was digested with trypsin (Sigma); the other half digested with chymotrypsin (Sigma). The ratio of protein:protease was 50:1 and the digest was carried out for four hours at 37°C. A second, equivalent amount of protease was added and the solutions digested as before. The reaction was then

stopped by lyophilisation of the digested protein.

### 2.6.3 Purification of peptides using HPLC

The lyophilised digested protein, obtained as described in Section 2.6.2, was resuspended in 200µl sterile double deionised distilled water, transferred to acid-washed glass test tubes and centrifuged at 1000xg<sub>av</sub> for 2 minutes to remove particulate matter. The sample was then applied to the C18 reverse phase column of the high pressure liquid chromatography (HPLC, Beckman) system using a pH2 trifluoroacetic acid buffering system. The peptides were eluted with a gradient of 0-70% acetonitrile over 60 minutes. Only the peptides which appeared as well-defined, distinct peaks on the trace obtained were collected, then lyophilised and stored at -20°C until required.

The lyophilised peptides were redissolved in 500µl double deionised distilled water. The purity of the peptides was checked by applying a sample of each individual peptide to the same HPLC column as used previously with a 1mM sodium phosphate pH6.2 buffering system. The peptide was again eluted from the column with a 0-70% acetonitrile gradient. The peptides on the trace which appeared to be greater than 90% pure were lyophilised and then taken to Aberdeen University to be sequenced by Mr. B. Dunbar on the SERC funded protein sequencing facility.

## 2.7 IMMUNOLOGICAL METHODS

### 2.7.1 Preparation of antiserum

250 $\mu$ g ribonuclease inhibitor was resolved on a preparative 10% (w/v) polyacrylamide slab gel (Section 2.4.6), then stained for protein using Coomassie Brilliant Blue R (Section 2.4.5.A). After destaining, the gel was washed with distilled water for 30 minutes to remove excess acid. The protein band corresponding to ribonuclease inhibitor was excised from the gel with a scalpel, diced, frozen in liquid N<sub>2</sub> and ground to a fine powder using a mortar and pestle.

The powdered material was sonicated with 0.5ml 0.9% (w/v) NaCl and 1ml Freund's complete adjuvant and this mixture was injected subcutaneously at multiple sites on the back of a New Zealand white rabbit. Additional booster injections were administered in Freund's incomplete adjuvant 2 weeks before each bleeding.

Antiserum was collected by bleeding the rabbit from a marginal ear vein. After allowing the blood to clot overnight at 4°C, the antiserum was removed with a pasteur pipette, clarified by centrifugation at 2,000 rpm (1,000xg<sub>av</sub>) in a Beckman benchtop centrifuge for 10 minutes, aliquoted into 1ml fractions and stored at -20°C.

### 2.7.2 Isolation of IgG from immune serum

IgG was purified from antiserum as described by Johnstone and Thorpe (1982).

20ml immune serum was warmed to 25°C in a water bath. Solid sodium sulphate was added to make a 18% (w/v) solution with stirring

to allow the salt to dissolve. The solution was incubated at 25°C for 30 minutes, then centrifuged at 4,000 rpm ( $2,500 \times g_{av}$ ) for 30 minutes at 25°C in the HB4 rotor of a Sorvall RC-5 centrifuge. The supernatant fraction was discarded and the protein precipitate redissolved in 10ml water. This solution was warmed to 25°C and sodium sulphate added to make a 14% (w/v) solution, allowing for the salt carried over in the first precipitate. The solution was again incubated and centrifuged as described above. The resultant precipitate was resuspended in 5ml water and dialysed overnight against 0.07M sodium phosphate buffer pH6.3 at room temperature.

The dialysate was then applied to a 10ml DEAE-anion exchange column (Whatman DE52) which had been equilibrated with 0.07M sodium phosphate buffer pH6.3. 1ml samples were collected and the absorbance at 280nm monitored. The starting buffer was used to elute IgG in a single asymmetric peak of which the earliest fractions contained the purest IgG.

### 2.7.3. Western blotting

Immunoblots were performed using a modification of Towbin *et al.* (1979).

Proteins were resolved on SDS PAGE (Section 2.4.3) and electrophoretically transferred onto nitrocellulose paper (Schleicher and Schuell) at 70V for 3 hours using the Bio-Rad Trans-blot<sup>TM</sup> apparatus. The transfer buffer consisted of 0.025M Tris, 0.192M glycine, 0.02% (w/v) SDS, 20% (v/v) methanol with an approximate pH of 8.3.

After transfer of the proteins to nitrocellulose membrane, additional binding sites on the paper were saturated by incubating the filters overnight at 4°C in buffer A (TBS (Table 2.4 ) and 3% (w/v)

reconstituted dried skimmed milk) supplemented with 0.5% (v/v) Tween 20 (Sigma). Immune serum, at a dilution of 1:200 in buffer A supplemented with 2% (v/v) heat inactivated goat serum (SAPU), was incubated with the filters for 2 hours at room temperature with shaking. Excess free antibody was removed by washing the nitrocellulose filters with TBS for 15 minutes, then TBS made 0.05% with Tween 20 for 15 minutes, followed by a further 15 minutes wash with TBS. In general, antibody-antigen complexes were detected using donkey anti-rabbit IgG conjugated to horseradish peroxidase (SAPU) by incubating the filters with a solution of the conjugate diluted 1 in 100 with buffer A. After 2 hours, the membrane filters were washed as previously described. The immunoblot was then allowed to develop in developing solution (TBS containing 2.2mM 4-chloro-1-naphthol (Sigma) and 0.03% (v/v) H<sub>2</sub>O<sub>2</sub>) for 15-20 minutes, rinsed in distilled water and allowed to dry.

Occasionally, when greater sensitivity was required, a western blot was developed with the biotin-streptavidin detection system (Amersham). Briefly, after incubation with immune serum, the filters were incubated with a 1 in 500 dilution of donkey anti-rabbit IgG conjugated to biotin for 2 hours at room temperature, then washed as described above. The biotin was then detected by incubating the membranes with a 1 in 400 dilution of streptavidin-horseradish peroxidase for 30 minutes, followed by 6x5 minute washes in TBS. The blot was then developed as described above.

#### 2.7.4 Enzyme linked immunosorbent assay (ELISA)

Antibody levels in samples of serum were measured using the ELISA system as described by Campbell (1984)

Antigen, in 100 $\mu$ l PBS (Table 2.4) was bound to the wells of a Dynatech microtitre plate by incubating overnight at 4°C. Excess antigen was removed by washing the plate with PBS containing 0.05% (v/v) Tween 20 (Sigma). Residual binding sites were blocked for 2 hours at room temperature with 10 mg/ml BSA (fraction V, Sigma) in PBS. The plate was rinsed with buffer as before, then serial dilutions of antiserum (1/100 to 1/100,000) in 100 $\mu$ l PBS were placed in the wells. After incubating for 2 hours at room temperature, the plate was washed with PBS containing 0.05% (v/v) Tween 20. The plates were then incubated for 1 hour at room temperature with 100 $\mu$ l donkey anti-rabbit IgG conjugated to horseradish peroxidase (SAPU) which had been diluted 1 in 1000 with PBS containing 0.5% (v/v) Tween 20. Excess conjugate was removed by washing the plate with PBS containing 0.05% (v/v) Tween 20. The quantity of peroxidase activity bound to each well was determined by incubating with 100 $\mu$ l substrate solution (0.04% (w/v) o-phenylenediamine (Sigma) 0.04% (v/v) H<sub>2</sub>O<sub>2</sub>, 36mM citric acid, 128mM NaH<sub>2</sub>PO<sub>4</sub> pH6) in the dark for 30 minutes at room temperature. The reaction was terminated by the addition of 50 $\mu$ l 2M H<sub>2</sub>SO<sub>4</sub>. The absorbance at 492nm was determined using a Titertek multiscan spectrophotometer.

The titre of immune serum was determined by finding the dilution at which immune serum and pre-immune serum from the same animal gave an equal absorbance at 492nm.

#### 2.7.5 Immunoabsorption

The anti-serum raised against ribonuclease inhibitor was found to recognise other liver proteins in addition to inhibitor. To improve the specificity of the anti-serum, the contaminating antibodies were

adsorbed from the serum by passing it through an affinity column which consisted of liver proteins depleted in ribonuclease inhibitor bound to Sepharose 4B.

Approximately 20 mg of protein from the run-through fraction of the RNase A-Sepharose affinity column (Section 2.5.2) was covalently bound per ml of activated Sepharose 4B (Sigma) as described in Section 2.5.1. 10mls immune serum was passed through a 10ml bed of conjugated resin at a flow rate of 10ml/hour at room temperature. 1ml fractions were collected and monitored for specificity by ELISA (Section 2.7.4) and western blotting (Section 2.7.3). The column was regenerated by eluting bound antibody with 0.5M ammonium hydroxide containing 3M potassium thiocyanate.

#### 2.7.6 Removal of *E.Coli* antibodies from immune serum.

Immune serum often contains antibodies which bind to *E.Coli* antigens and this may cause a high background when screening an expression library with immune serum. These contaminating *E.Coli* antibodies can be removed by incubating the immune serum with a bacterial lysate bound to a nitrocellulose filter (Huynh *et al.*, 1985).

A crude cell lysate was prepared from a non-recombinant  $\lambda$ gt11/Y1089 lysogen as described in Section 2.11.2. This crude cell lysate contains many *E. Coli* proteins, including that for  $\beta$ -galactosidase. All of the following steps were carried out at room temperature. The lysate was incubated with 140mm diameter nitrocellulose filter discs (Schleicher and Schuell) for 15 minutes to allow the proteins to bind to the nitrocellulose. The filter discs were then washed for 5 minutes with TBS (Table 2.4). IgG, purified from



immune serum (Section 2.7.2), was incubated with 2 separate filters for 10 minutes to remove contaminating *E. Coli* antibodies.

## 2.8 CLONING METHODS

The following section describes the library which was screened in this project, the plasmid which was used in the generation of subclones and the bacterial strains which were required.

### 2.8.1 Rat Liver $\lambda$ gt11 Library

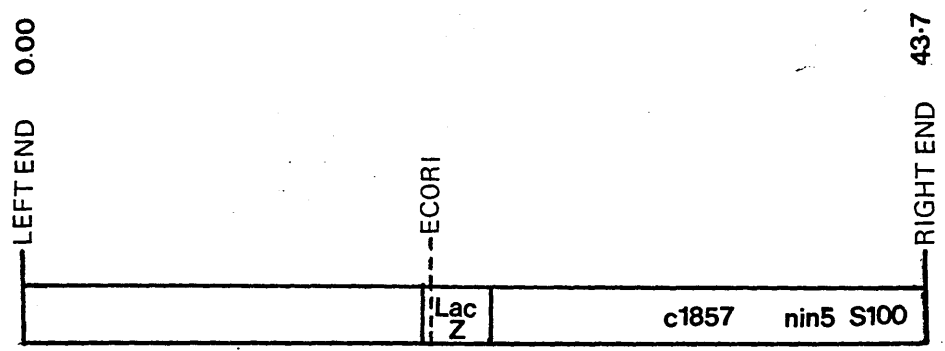
The library which was screened in this project was a rat liver cDNA library in the expression vector  $\lambda$ gt11. The preparation of this library is described by Schwarzbauer *et al.* (1983) and was kindly donated by R. Hynes.

The structure of the expression vector  $\lambda$ gt11 (*lac* 5, *c1857*, *nin* 5, S100) is shown in Figure 2.1.  $\lambda$ gt11 contains a temperature sensitive repressor of lytic growth (*c1857*) that is inactive at 42°C and active at 32°C allowing a selection against lysogenic growth. The amber mutation S100 renders the phage induced lysis defective in *E.Coli* hosts which lack the suppressor *sup F*. Foreign DNA is inserted into a unique EcoRI cleavage site located within the *lac Z* gene, near the COOH terminus of  $\beta$ -galactosidase. Foreign DNA in this vector can be expressed as a fusion protein with  $\beta$ -galactosidase, assuming the inserted DNA is in the correct frame and orientation. Recombinant libraries in  $\lambda$ gt11 can thus be screened with antibodies as an antibody may detect the foreign antigen encoded by the DNA insert which is fused to  $\beta$ -galactosidase.

There are many problems associated with the expression of foreign

## FIGURE 2.1 STRUCTURE OF EXPRESSION VECTOR $\lambda$ GT11

The structure of the expression vector  $\lambda$ gt11 (*lac* 5, c1857, nin 5, S100) is outlined. Also shown is the position of the unique EcoRI site in the *lac Z* gene into which foreign cDNA sequences are inserted. The transcriptional orientation of *lac Z* is indicated by the horizontal arrow and the size of the vector is shown in kilobase pairs. The salient features are described in detail in Section 2.8.1



←  
3'... Transcription .. 5'

DNA in *E.Coli* e.g. the foreign protein is often unstable and the presence of these novel proteins may be toxic to the host cell.

However, these problems have been minimised by the use of bacterial hosts which allow the expression and accumulation of foreign proteins.

These host strains are described more fully in Section 2.8.3

### 2.8.2 Plasmid, pTZ

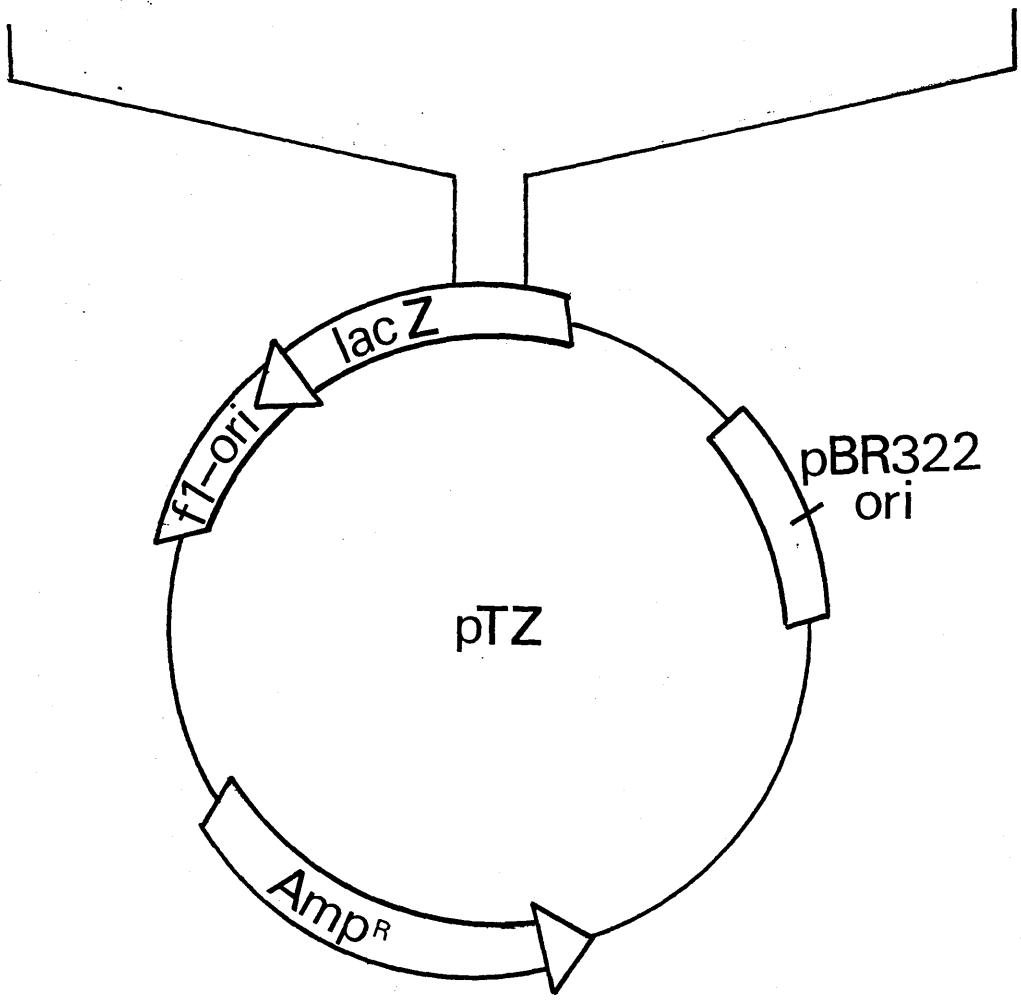
The positive clones obtained from the  $\lambda$ gt11 cDNA library were subcloned into the multi-functional vector pTZ (Pharmacia) to permit dideoxy DNA sequencing and also for preparative growth to obtain probes for northern blotting. A diagram of pTZ is shown in Figure 2.2.

pTZ vectors contain the *lac Z* gene and within this gene is the polylinker region from pUC 18 or 19 into which a cDNA insert can be cloned. When pTZ is introduced into *lac Z*<sup>-</sup> *E.Coli* strains, the plasmid gives rise to blue colonies on agar plates supplemented with IPTG (isopropyl  $\beta$ -D thiogalactopyranoside, Sigma) and X gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D galactoside, BRL) i.e.  $\beta$ -galactosidase activity is restored to the bacterial strain by a process known as  $\alpha$ -complementation. The active  $\beta$ -galactosidase converts the colourless substrate X gal to a blue pigment which gives the characteristic blue colour to the bacterial colonies. When DNA fragments are cloned into the polylinker region, the *lac Z* gene is disrupted and will thus fail to complement *lac Z*<sup>-</sup> *E.Coli* strains. The recombinant plasmid transformants remain *lac Z*<sup>-</sup> and therefore cannot metabolise X gal to a blue colour. Resulting colonies, grown on agar plates supplemented with X gal and IPTG, will remain white. To distinguish these colonies from untransformed host, the transformation mix is grown in the

**FIGURE 2.2 STRUCTURE OF PLASMID, pTZ**

The features of the multifunctional plasmid pTZ (Pharmacia) are outlined and are described in detail in Section 2.8.2.

Polylinker pUC18/19	T7 Promoter	Reverse Primer CAGGAAACAGCTATGAC
------------------------	-------------	--



presence of ampicillin as this prevents growth of untransformed host. pTZ transformed colonies are able to grow as the plasmid contains an ampicillin resistance gene. Thus, a white colony obtained from an agar plate containing ampicillin and supplemented with IPTG and X gal should contain recombinant plasmid carrying the insert of interest.

The plasmid pTZ contains both the bacteriophage f1 and the pBR322 origins of replication for generation of single and double stranded DNA. Single stranded DNA can be used for dideoxy DNA sequencing. The production of single stranded DNA requires the pTZ transformed cells to be superinfected with M13K07 helper phage. These phages provide the replication enzymes and packaging proteins to synthesise and package the single stranded pTZ DNA. The M13K07 itself is not efficiently replicated and packaged when competing with pTZ, so that the ratio of single stranded DNA isolated from pTZ and M13K07 co-transfected cells is at least 50:1.

### 2.8.3 Bacterial Strains

The bacterial strains utilised in this project are outlined in Table 2.2. *E. Coli* Y1088, Y1089, Y1090 were the strains required for cloning in  $\lambda$ gt11 and are described by Young and Davis (1983) .

The features which make these strains suitable for screening  $\lambda$ gt11 cDNA expression libraries include the following:

Y1088 is *hsd R*<sup>-</sup> and *hsd*<sup>+</sup> i.e. it is defective for host-controlled restriction and modification. Thus, this strain is suitable for amplification of the library and also for determining the titre of bacteriophage stocks (Section 2.10.4)

Y1089 contains a mutation in the *hfl A* gene which favours the production of lysogens (Section 2.11.2). Also, Y1089 lacks the *sup F*

**TABLE 2.2 GENOTYPE OF BACTERIA UTILISED IN CLONING,  
SUBCLONING AND SEQUENCING PROCEDURES**

This table describes the genotype of the various bacteria used while carrying out this project and are described in more detail in Section 2.8.3



<u>STRAIN</u>	<u>GENOTYPE</u>	<u>REFERENCE</u>
<u>Y1088</u>	<i>supE</i> , <i>supF</i> , <i>metB</i> , <i>trpR</i> , <i>hsdR</i> <sup>-</sup> <i>hsdM</i> <sup>+</sup> <i>tonA21</i> , <i>strA</i> , $\Delta$ <i>lacU169</i> , <i>proC</i> ::Tn5(pMC9)	Young and Davis (1983b)
<u>Y1089</u>	<i>proA</i> <sup>+</sup> , <i>araD139</i> , <i>strA</i> , <i>hflA</i> , 150[chr::Tn10] $\Delta$ <i>lacU169</i> , $\Delta$ <i>lon</i> , (pMC9)	Young and Davis (1983b)
<u>Y1090</u>	<i>proA</i> <sup>+</sup> , <i>araD139</i> , <i>strA</i> , <i>supF</i> , [ <i>trpC22</i> ::Tn10] $\Delta$ <i>lacU169</i> , $\Delta$ <i>lon</i> , (pMC9)	Young and Davis (1983b)
<u>DS941</u>	AB1157, <i>rec F</i> <sup>-</sup> , <i>lac Z</i> M15, <i>lac I</i> <sup>q</sup>	Sherratt,D. Dept. of Genetics, Glasgow University
<u>MV1190</u>	$\Delta$ ( <i>lac-pro</i> ), <i>thi</i> , <i>supE</i> , $\Delta$ ( <i>sr1-recA</i> )::Tn10( <i>tet</i> <sup>r</sup> ), F': <i>traD36</i> , <i>proAB</i> , <i>lacI</i> <sup>q</sup> $\Delta$ M15	Bio-Rad

gene which is required to suppress the amber mutation in the S gene of  $\lambda$ gt11 and hence this bacterium is unable to lyse until the addition of chloroform. Therefore, preparative amounts of the induced fusion protein can be produced from lysogens.

Y1090 is the plating host for screening  $\lambda$ gt11 libraries (Section 2.10.1). It contains the *sup F* gene and therefore the bacterium will lyse in the presence of bacteriophage. Thus  $\lambda$ gt11 libraries are screened as plaques with antibody probes.

Y1089 and Y1090 lack the *lon* protease, an enzyme which degrades foreign proteins in *E. Coli*. Thus the stability of the induced recombinant fusion protein is increased.

Finally, all three strains contain the plasmid pMC9. This is a plasmid derived from pBR322 which contains the *lac I $\phi$*  gene. This gene codes for the repressor protein which binds to the operator region of the *lac* operon thus preventing transcription and hence expression of  $\beta$ -galactosidase. Therefore, the  $\beta$ -galactosidase fusion protein need not be expressed during infection and early growth of the phage. This is advantageous as the presence of foreign proteins can be toxic to the bacterial cell. When required, this repression can be overcome by the addition of IPTG, a gratuitous inducer of the *lac* operon which induces the expression of the  $\beta$ -galactosidase fusion protein.

DS941 was the recommended bacterial strain for transformation with recombinant pTZ subclones.

MV1190 was the bacterial strain used to produce single stranded DNA for use in dideoxy DNA sequencing. This bacterium has the F' plasmid which is required for the superinfection of a defective M13 phage (M13KO7). The plasmid also carries a gene coding for an enzyme involved in proline synthesis which is defective in the host

cell. Selection by growth on minimal medium agar plates can thus be used to ensure that the plasmid encoding the male pilus is maintained.

## 2.9 GENERAL CLONING PROCEDURES

### 2.9.1 Storage of plasmid and lambda DNA

Plasmid DNA was stored in TE buffer (Table 2.4) at  $-20^{\circ}\text{C}$  in Eppendorf tubes. Lambda DNA was stored at  $4^{\circ}\text{C}$  in TE buffer.

### 2.9.2 Storage of bacterial strains

For short term storage, the bacterial strains were streaked out onto an agar plate containing the appropriate antibiotic. The plates were grown overnight at  $37^{\circ}\text{C}$ , then sealed with Nescofilm and stored at  $4^{\circ}\text{C}$  for 1-2 weeks.

For long term storage, 0.15ml glycerol was added to 0.85ml of an overnight culture grown in LB medium plus the correct antibiotic. The culture was then snap frozen in a dry ice-methanol bath and stored at  $-20^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$ . Bacteria stored in this manner remained viable for the duration of this project.

### 2.9.3 Composition of growth media

The composition of growth media is shown in Table 2.3. All media were autoclaved at 15 lb p.s.i. for 20 minutes. If required, antibiotics were added aseptically to the medium when it had cooled to approximately  $50^{\circ}\text{C}$ .

**TABLE 2.3 COMPOSITION OF GROWTH MEDIA**

The compositions of the different media used throughout the project are shown. All media were autoclaved prior to use (Section 2.2) and antibiotics, if required, were added aseptically when the media had cooled to approximately 50°C (Section 2.9.4)

## **A. LIQUID MEDIA**

LB: 10g bacto tryptone (Difco), 5g yeast extract(Difco), 5g NaCl per 1000ml.

2xTY: 16g bacto tryptone, 10g yeast extract, 5g NaCl per 1000ml.

### Minimal Medium

The following reagents were autoclaved separately and cooled before mixing aseptically:

900ml water

100ml 10xM9 salt (337mM Na<sub>2</sub>HPO<sub>4</sub>, 220mM KH<sub>2</sub>PO<sub>4</sub>, 187mM NH<sub>4</sub>Cl, 85mM NaCl)

1ml 1M MgSO<sub>4</sub>

1ml 0.1M CaCl<sub>2</sub>

1ml 1M thiamine HCl

10ml 20% (w/v) glucose

## **B. Media containing agar**

LB agar: medium as for LB medium plus 1.5% (w/v) Bacto agar (Difco).

Top agar: medium as for LB medium plus 0.5% (w/v) Bacto agar.

Minimal agar: medium as for minimal medium plus 1.5% (w/v) Bacto agar.

#### 2.9.4 Supplements to growth media

Stock solutions were made up in sterile water to the appropriate concentration, filter sterilised using a Millipore filter (0.2µm) and stored at -20°C.

Ampicillin (Sigma) and Kanamycin (Sigma) were made at stock concentrations of 50mg/ml. The working concentration of Ampicillin was 100µg/ml whereas the working concentration of Kanamycin was 70µg/ml.

#### 2.9.5 Commonly used solutions for nucleic acid procedures

The solutions used most frequently in cloning procedures are listed in Table 2.4. All solutions used in the following sections were autoclaved at 15 lb p.s.i. for 20 minutes before use.

#### 2.9.6 Organic reagents

Crystalline phenol was redistilled at 160°C and stored in aliquots at -20°C. When required, aliquots were melted and made 0.1% (w/v) with respect to 8-hydroxyquinoline, an antioxidant. The melted phenol was extracted with an equal volume of 0.1M Tris.HCl pH7.5 and stored saturated in the same buffer in the dark at 4°C. Phenol was also extracted and stored saturated with TE buffer (Table 2.4) and with chloroform:isoamyl alcohol (24:1).

TABLE 2.4                    COMMONLY USED SOLUTIONS

The solutions used most frequently are outlined in this table. All solutions were sterilised by autoclaving as described in Section 2.2. Solutions which were used in procedures involving RNA were first treated with diethylpyrocarbonate (DEPC, Sigma) and then autoclaved to prevent contamination with ribonucleases.

Phage buffer (SM) 50mM Tris.HCl pH7.5, 100mM NaCl, 10mM MgSO<sub>4</sub>, 0.01% (w/v) gelatin.

Phosphate buffered saline (PBS) 140mM NaCl, 2.7mM KCl, 1.5mM KH<sub>2</sub>PO<sub>4</sub>, 1.8mM Na<sub>2</sub>HPO<sub>4</sub> pH7

TE 10mM Tris.HCl pH8, 1mM EDTA.

Tris buffered saline (TBS) 50mM Tris.HCl pH7.5, 150mM NaCl

10X SSC 1.5M NaCl, 0.15M trisodium citrate pH7

50X Denhardt's solution 1% (w/v) BSA, 1% (w/v) Ficoll type 400  
1% (w/v) polyvinylpyrrolidone pH7

20X SSPE 3.6 M NaCl, 200mM NaH<sub>2</sub>PO<sub>4</sub> pH7, 20mM EDTA

10X TBE 0.89M Tris-borate, 0.89M boric acid, 0.02M EDTA



### 2.9.7 Agarose gel electrophoresis

DNA fragments were separated by horizontal gel electrophoresis using a TBE buffering system as described by Maniatis *et al.*, 1982. The electrophoresis buffer contained 0.089M tris-borate, 0.089M boric acid 0.002M EDTA.

1.5% (w/v) agarose gels were routinely used for visualising the cDNA inserts from restriction digestion of recombinant lambda or plasmid DNA. 1% (w/v) agarose gels were used for monitoring lambda or plasmid DNA during their purification.

Agarose gels were prepared by autoclaving for 5 minutes at 5lb p.s.i. the correct amount of agarose in the desired quantity of electrophoresis buffer. The agarose solution was cooled to 55°C, ethidium bromide (10mg/ml) added to a final concentration of 0.5µg/ml and the gel poured on a flat surface.

DNA samples were prepared by the addition of 0.1 volumes of sample buffer containing 0.25% (w/v) bromophenol blue in 40% (w/v) sucrose.

Electrophoresis was performed using either BRL H5 or H6 apparatus. The gel was immersed in the electrophoresis buffer containing 0.5µg/ml ethidium bromide. The DNA samples were applied to the pre-formed wells and electrophoresed until the tracking dye had reached the bottom of the gel. DNA was visualised by ethidium bromide fluorescence on a transilluminator. Gels were photographed with a Polaroid CU-5 camera using a 665 positive/negative film.

## 2.10 ISOLATION OF CLONES FROM A cDNA EXPRESSION LIBRARY IN $\lambda$ GT11

### 2.10.1 Preparation of plating bacteria

*E. Coli* strains Y1088, Y1089 and Y1090 were prepared for plating bacteriophage by isolating a single colony of *E. Coli* from an LB agar plate supplemented with 100 $\mu$ g/ml Ampicillin (Sigma), inoculating 10ml LB containing 0.2% (w/v) maltose (Sigma) and incubating at 37°C overnight. Bacteria grown in the presence of maltose absorb bacteriophage lambda more efficiently as the sugar induces the maltose operon which contains the gene coding for the lambda receptor. The bacteria were then harvested in a Beckman benchtop centrifuge at 3000 rpm (1,500xg<sub>av</sub>) for 10 minutes at room temperature. The medium was decanted and the bacterial pellet resuspended in 5ml 10mM MgSO<sub>4</sub>.

### 2.10.2 Screening of a cDNA expression library with polyclonal antibodies

The library screened in this project was a rat liver cDNA library in the expression vector  $\lambda$ gt11. The library is described in more detail in Section 2.8.1.

For the initial screen, 0.6ml of Y1090 plating bacteria (Section 2.10.1) was mixed with 3x10<sup>5</sup> p.f.u. of the library and incubated at 37°C for 15 minutes to allow the phage to adsorb to the bacteria. 7ml top agar (Table 2.3) was added and the culture poured onto a well-dried 140mm LB plate supplemented with 10mM MgSO<sub>4</sub>. The agar was allowed to harden, the plate was inverted and incubated at 42°C until the plaques were just visible (approximately 2 hours). The plate was

then overlaid with a dry nitrocellulose filter (Schleicher and Schuell) which had been previously soaked in 10mM isopropyl  $\beta$ -D thiogalactopyranoside (IPTG, Sigma) and the plate was incubated for a further 3 hours at 37°C. The plate was removed to room temperature and the position of the filter on the plate was marked using a needle. If a duplicate filter was required, a second filter was overlaid after the first had been removed and the plate returned to incubate at 37°C for a further 3 hours. The filter, which had been carefully removed from the plate, was placed in a petri dish containing 15ml TBS (Table 2.4) . This and all subsequent steps were carried out at room temperature. The filter was incubated with 15ml TBS containing 2% (w/v) BSA (fraction V, Sigma) for 15 minutes. It was then incubated overnight with IgG purified from immune serum (Section 2.7.2) from which *E. Coli* antibodies had been removed (Section 2.7.6), at a concentration of 50  $\mu$ g IgG/ml in 15ml TBS containing 2% (w/v) BSA and 0.05% (v/v) Tween 20 (Sigma). The antibody-antigen complexes were detected using the donkey anti-rabbit IgG conjugated to horseradish peroxidase (SAPU) system and the filters developed as described in Section 2.7.3.

Due to the high density of the plaques in this initial screen, it was impossible to unambiguously identify the plaques giving rise to candidate positive signals on the filter. Hence, it was necessary to isolate the plaques from the general area of the positive signal. Bacteriophage were then isolated from these plaques (described in Section 2.10.3) and then taken through two successive rounds of antibody screening at progressively lower plaque densities. The second round of screening was performed with  $1 \times 10^4$  p.f.u. of purified phage and 200 $\mu$ l of Y1090 which were plated onto 90mm LB, 10mM MgSO<sub>4</sub>

plates using 3ml top agar. The plaques obtained from the second round of screening were discrete and thus it was possible to isolate individual positive plaques. The third and final round of screening utilised 500 p.f.u. of bacteriophage with 200µl Y1090 as described for the second round of screening. Each plaque gave rise to a positive signal, hence further purification was unnecessary.

### 2.10.3 Purification of bacteriophage from a positive plaque

Plaques of interest were isolated using the broad end of a sterile pasteur pipette which was stabbed through a plaque into the agar below. The agar plug containing a plaque was removed from the end of the pasteur pipette with the aid of a sterile toothpick into an Eppendorf tube containing 1ml SM buffer (Table 2.4) and 1 drop of chloroform. The bacteriophage particles were allowed to diffuse out of the agar for 12-16 hours at 4°C. The Eppendorf was then centrifuged in an Anderman 4515 microfuge for 2 minutes. The supernatant fraction containing the bacteriophage particles was transferred to a fresh sterile Eppendorf tube and stored at 4°C. The titre of phage particles was determined as described in Section 2.10.4. In general, a single plaque yielded approximately  $10^7$  p.f.u./ml.

### 2.10.4 Determination of the titre of bacteriophage lambda

Y1088 plating cells were prepared as described in Section 2.10.1. 200µl of plating cells were mixed with 3ml top agar (Table 2.3) and poured onto a 90mm LB plate (Table 2.3). When the top agar had set, the plate was inverted and grown at 37°C until a lawn of bacteria was visible. The bacteriophage to be titred were diluted in SM buffer (Table

2.4) and 25µl of the diluted phage was dropped onto one quarter of the LB plate containing the lawn of Y1088, thus four separate dilutions were applied to a single plate. The drop of SM buffer containing the bacteriophage was allowed to dry, then the plates were inverted and incubated overnight at 37°C. The following morning, the number of plaques present in each quarter could be counted and thus the number of plaque forming units (p.f.u.) per millilitre could be calculated.

2.10.5 Preparation of a high titre lysate

High titre lysates were prepared from plate stocks of the purified positive phage obtained from the third round of screening (Section 2.10.2) as described by Maniatis *et al* (1982). A single positive plaque was picked using a sterile toothpick and washed into a test tube containing 200µl of plating bacteria Y1088 (Section 2.10.1). The phage and bacteria were incubated for 15 minutes at 37°C, 3ml of top agar were added and the culture poured onto a 90mm LB plate supplemented with 10mM MgSO<sub>4</sub>. The plate was then incubated at 42°C until plaques were visible. At this stage, 2ml SM buffer (Table 2.4) was added to the plate which was then incubated overnight at 4°C. The SM buffer containing the high titre lysate was removed from the plate and centrifuged in the HB4 rotor of the Sorvall centrifuge at 8,000 rpm (10,000xg<sub>av</sub>) for 15 minutes at 4°C to remove bacterial debris. The supernatant fraction was transferred to sterile universals with the addition of a few drops of chloroform.

Bacteriophage stored in this manner are stable for many years with the titre of phage dropping by a factor of 10 per year. In general, a high titre lysate yielded approximately 10<sup>10</sup> p.f.u./ml.

## 2.11 CHARACTERISATION OF LAMBDA cDNA CLONES

The methods described in this section are described by Huynh *et al.* (1985).

### 2.11.1 Preparation of lysogens

*E. Coli* strain Y1089 (Section 2.8.3) was infected with each clone at a multiplicity of infection of 5. The correct proportion of phage and bacteria were incubated at room temperature for 1 hour. The culture was then diluted 1:100, 1:1000 and 1:10,000 in TBS (Table 2.4 ) and 100 $\mu$ l of each dilution was spread onto 90mm LB plates which were incubated at 30°C overnight. The plate which contained discrete colonies was then replica-plated with the aid of a pedestal and sterile Whatman filter discs as follows: the plate containing the discrete colonies was pressed onto the top filter on the pedestal thus transferring the colonies to the filter. A fresh LB plate was then placed on top of the filter and thus a replica of the original plate was obtained on the new plate. This procedure was repeated so that two plates which were replicas of the original plate were obtained. The first plate was incubated at 42°C while the second plate was incubated at 30°C until colonies were visible. Colonies which grew at 30°C (a temperature at which the temperature-sensitive phage repressor is functional) but not at 42°C were considered to be lysogenic. To ensure that individual colonies were lysogenic, a single colony was streaked out onto a fresh LB plate, incubated at 30°C and then replica-plated. If this colony continued to grow at 30°C but not at 42°C then lysogeny was assured.

### 2.11.2 Preparation of a crude lysate from a $\lambda$ gt11 recombinant lysogen

10ml LB medium (Table 2.3) was inoculated with a single colony of Y1089 recombinant lysogen (Section 2.11.1) and incubated at 30°C overnight. 100ml of pre-warmed LB medium was inoculated with 1ml of overnight culture and incubated at 30°C until an optical density of 0.5 at 600nm was obtained. The cells were then heat-shocked for 20 minutes at 45°C and isopropyl  $\beta$ -D thiogalactopyranoside (IPTG, Sigma) was added to a final concentration of 10mM to induce *lac z* - directed production of the fusion protein. The cells were incubated at 37°C for a further 3 hours and then harvested by centrifugation at 8,000 rpm (10,000 $\times$ g<sub>av</sub>) in a Sorvall GSA rotor for 15 minutes. The cell pellet was resuspended in 2ml TBS (Table 2.4), transferred to a sterile Corex tube and the cells lysed by repeated freezing in a dry ice-methanol bath and thawing in a 37°C water bath. Bacterial DNA was digested by the addition of 10 $\mu$ g DNase I and the suspension centrifuged at 8,000 rpm (10,000 $\times$ g<sub>av</sub>) for 15 minutes in a Sorvall HB4 rotor. The resulting supernatant fraction contained the fusion protein.

### 2.12 ISOLATION OF LAMBDA AND PLASMID DNA

All the procedures in the following section are described in Maniatis *et al* (1982).

Purified DNA was analysed by agarose gel electrophoresis (Section 2.9.7) and the concentration of DNA was determined by measuring the optical density at 260nm (Section 2.12.5)

### 2.12.1 Preparation of lambda DNA from lysogens

A lysogenic colony was isolated (Section 2.11.1) and used to inoculate 10ml LB medium (Table 2.3) in a universal and grown overnight at 30°C. A two litre flask, containing 400ml pre-warmed LB medium, was inoculated with 4ml of the overnight culture and grown at 30°C until an optical density of 0.5 at 600nm was reached. At this point, the culture was heat-shocked at 45°C for 20 minutes and then incubated at 37°C for a further 3 hours. The bacterial cells were then harvested by centrifugation at 8,000 rpm (10,000 $xg_{av}$ ) in a Sorvall GSA rotor for 15 minutes at 4°C. The bacterial pellet was resuspended in 10ml 10mM Tris.HCl pH 7.4, 50mM NaCl, 5mM MgCl<sub>2</sub> and transferred to sterile Corex tubes. The cells were lysed by the addition of 5 drops of chloroform to the bacterial suspension and incubated at room temperature for 30 minutes. Cell lysis was evident due to the appearance of bacterial DNA which made the suspension viscous. To remove bacterial nucleic acids, DNase I (Sigma) and RNase A (Sigma) were added to a final concentration of 1 $\mu$ g/ml and the cell lysate was incubated for 30 minutes at room temperature. Solid NaCl and PEG 6000 were added to the lysate to a final concentration of 1M and 10% (w/v) respectively. The cell lysate was then incubated overnight at 4°C to allow the phage to precipitate. The phage were recovered by centrifugation at 8,000 rpm (10,000 $xg_{av}$ ) for 10 minutes at 4°C in a Sorvall HB4 rotor. The supernatant fraction was discarded and the pellet resuspended in 5ml SM buffer (Table 2.4). An equal volume of chloroform was added, vortexed for 30 seconds and centrifuged in a Sorvall HB4 rotor at 3,000 rpm (1,500 $xg_{av}$ ) for 15 minutes at 4°C. The aqueous phase was recovered and the volume measured. Caesium chloride was added to a concentration of 0.75 mg/ml, the solution



gently mixed and transferred to Beckman SW.50 tubes. The remainder of the tube was filled with SM buffer containing 0.75mg/ml caesium chloride. This suspension was centrifuged at 45,000 rpm (137,000 $\times g_{av}$ ) at 4°C overnight. After centrifugation, a pale band was visible when the tube was placed in front of a dark background. The band, containing intact phage particles, was isolated using a Gilson p200 pipette, transferred to dialysis tubing and dialysed at room temperature for 3 hours against 1000x volumes 50mM Tris.HCl pH8, 10mM NaCl, 10mM MgCl<sub>2</sub> with several changes of buffer. The phage were transferred to Eppendorf tubes and EDTA was added to a final concentration of 20mM. At this time, proteinase K (Sigma) and SDS were also added to final concentrations of 50 $\mu$ g/ml and 0.5% respectively. The solution was mixed gently by inverting the tube several times and was incubated for 1 hour at 65°C. This results in the digestion of the protein coat surrounding the lambda DNA. The DNA was extracted with phenol twice, with phenol:chloroform once, with chloroform:isoamyl alcohol (24:1) once and then finally ethanol precipitated (Section 2.12.4)

In general, a lambda purification resulted in a yield of approximately 100-150  $\mu$ g DNA.

### 2.12.2 Large scale preparation of plasmid DNA

This procedure was used to obtain large amounts of plasmid pTZ. A single colony of *E.coli* DS941 transformed with pTZ was used to inoculate 10ml LB medium (Table 2.3) supplemented with 100 $\mu$ g/ml Ampicillin (Sigma) and the medium incubated overnight at 37°C. 5ml of overnight culture was used to inoculate 500ml LB medium containing

the same concentration of Ampicillin as above. This culture was incubated at 37°C until an optical density of 0.4 at 600nm was obtained. 2.5ml of a 34mg/ml solution of chloramphenicol (Sigma) in ethanol was added to the culture and the incubation continued for approximately 12 hours. Chloramphenicol is an inhibitor of bacterial protein synthesis and thus stops genomic DNA synthesis, but not plasmid DNA replication. Therefore, the number of plasmid copies/cell is increased.

After the incubation, the bacterial cells were harvested by centrifugation at 8,000 rpm ( $10,000 \times g_{av}$ ) for 10 minutes at 4°C in the GSA rotor of a Sorvall RC-5 centrifuge. The supernatant fraction was discarded and the bacterial pellet washed in 100ml ice-cold STE (100mM NaCl, 10mM Tris.HCl pH7.8, 1mM EDTA). The pellet was resuspended in 10ml of buffer containing 50mM glucose, 25mM Tris.HCl pH8, 10mM EDTA and 5mg/ml lysozyme (Sigma). The suspension was then transferred to Beckman SW.27 polyallomer tubes and incubated at room temperature for 5 minutes, after which 20ml of freshly prepared 0.2M NaOH, 1% (w/v) SDS was added and the solution incubated on ice for a further 10 minutes. 15ml ice cold 5M potassium acetate pH4.8 was then added to the lysate and the contents of the tube mixed and left on ice for 10 minutes before centrifugation at 20,000 rpm ( $84,000 \times g_{av}$ ) for 20 minutes at 4°C to pellet the cellular DNA and the bacterial debris. The resultant supernatant fraction containing the plasmid was transferred to Corex tubes, 0.6 volumes isopropanol added and incubated at room temperature for 15 minutes. The DNA was recovered by centrifugation at 8,500 rpm ( $12,000 \times g_{av}$ ) for 30 minutes at room temperature. The supernatant fraction was discarded and the pellet washed with 70% (v/v) ethanol. The pellet was dried for 3 minutes in a vacuum desiccator and resuspended in 8ml TE

buffer (Table 2.4)

To obtain closed circular DNA, the plasmid was centrifuged in a caesium chloride-ethidium bromide gradient. 1g of solid caesium chloride was added per ml of resuspended pellet and mixed gently until all the caesium chloride was dissolved. 0.8ml of ethidium bromide (10mg/ml) was added per ml of caesium chloride solution. The resultant solution was transferred to Beckman SW.50 tubes and centrifuged at 45,000 rpm ( $137,000 \times g_{av}$ ) for 36 hours at 20°C. Closed circular plasmid DNA binds less ethidium bromide molecules than linear chromosomal, open circular and linearised plasmid DNA and hence has a higher buoyant density. It can therefore be seen as a separate band on the gradient and isolated.

Yields of approximately 1000µg of plasmid were routinely prepared by this method.

### 2.12.3 Small scale preparation of plasmid DNA

A single colony of *E.Coli* transformed with recombinant plasmid was inoculated into 5ml LB medium (Table 2.3) supplemented with 100µg/ml Ampicillin (Sigma) and grown overnight at 37°C. 1.5ml was transferred to an Eppendorf tube and centrifuged for 1 minute in an Anderman 4515 microfuge. The medium was removed and the pellet drained. The bacterial pellet was resuspended by vortexing in 100µl ice-cold 50mM glucose, 10mM EDTA, 25mM Tris.HCl, pH8.0 containing 5mg/ml lysozyme (Sigma) and was incubated at room temperature for 5 minutes. 200µl of freshly prepared 0.2M NaOH, 1% (w/v) SDS was added, the contents of the tube mixed by inversion and the Eppendorf incubated on ice for 5 minutes. 150µl of ice-cold 3M

potassium acetate pH4.8 was then added, the solution vortexed for 10 seconds and stored on ice for 5 minutes. The Eppendorf was centrifuged at 4°C and the supernatant fraction, which contained the plasmid DNA, was transferred to a fresh tube. The DNA was then phenol extracted and ethanol precipitated as described in Section 2.12.4. The resultant pellet was resuspended in 50µl TE buffer (Table 2.4) containing 20µg/ml DNase-free RNase.

#### 2.12.4 Phenol extraction and ethanol precipitation of DNA

Proteins were removed from DNA solutions by sequential extraction with phenol, phenol: chloroform and finally with chloroform:isoamyl alcohol (24:1)

A DNA-containing solution was phenol extracted by the addition of an equal volume of TE saturated phenol, vortexed for 30 seconds and centrifuged in an Anderman 4515 microfuge for 5 minutes at room temperature. The upper aqueous phase was carefully removed and transferred to a fresh Eppendorf tube. Extraction with phenol:chloroform and chloroform:isoamylalcohol (24:1) was performed in the same manner as for a phenol extraction.

DNA was precipitated with Burrough's absolute alcohol as follows: The volume of the DNA solution was estimated and the final concentration of monovalent ions adjusted to 0.25M by the addition of 2.5M sodium acetate pH5.2. Two volumes of ice-cold absolute alcohol were added and the solution mixed well. The DNA was allowed to precipitate at -20°C for at least one hour, then centrifuged for 10 minutes in a microfuge at 4°C. The alcohol was discarded and the pellet was dried in a vacuum desiccator for 2 minutes. Finally, the DNA was redissolved in TE buffer (Table 2.4).

#### 2.12.5 Determination of DNA concentration

The concentration of a DNA solution was estimated by obtaining the absorbance at 260nm. An optical density of 1 corresponds to approximately 50µg/ml for double stranded DNA and 40µg/ml for single stranded DNA.

### 2.13 SUBCLONING LAMBDA cDNA INSERTS INTO PLASMID pTZ

The plasmid vector pTZ (Section 2.8.2 and Figure 2.2) was used in the construction of all subclones in this project. The DNA to be subcloned was inserted into the EcoRI site in the polylinker region of the vector.

#### 2.13.1 Restriction digestion with EcoRI

Both plasmid and recombinant lambda DNA were digested with EcoRI (BRL) and agarose gel electrophoresis was performed to ensure that digestion of the plasmid was complete as undigested plasmid molecules may result in a high background of blue colonies upon transformation.

Restriction enzyme digests were generally carried out in a final volume of 20µl. A typical digestion mix contained the appropriate amount of DNA and EcoRI (1 unit of restriction enzyme digests 1µg DNA in 1 hour), 2µl x10 high salt buffer (100mM NaCl, 50mM Tris.HCl pH7.4, 10mM MgSO<sub>4</sub>, 1mM DTT) and the volume made up to 20µl with sterile water. The mix was incubated for 2 hours at 37°C and

the reaction terminated by heat denaturation. The digestion products were monitored by electrophoresis of a small aliquot on an agarose gel of the appropriate percentage (Section 2.9.7) Both plasmid and lambda DNA were extracted twice with phenol and then precipitated with ethanol as described in Section 2.12.2.

### 2.13.2 Alkaline phosphatase treatment of plasmid DNA

After digestion with EcoRI, the vector DNA was treated with alkaline phosphatase to remove the 5' phosphate groups, thus preventing recircularisation of the vector during the ligation reaction. Failure to do this may result in a high background of blue colonies upon transformation.

The digested plasmid DNA was resuspended in 20 $\mu$ l alkaline phosphatase buffer (10mM Tris.HCl, pH9.2, 0.1mM EDTA) and 0.5 $\mu$ l calf intestinal phosphatase (70 units/ $\mu$ l, Boehringer Mannheim) was added, mixed well and incubated at 37°C for 30 minutes. After incubation, the volume of sample was increased to 100 $\mu$ l with TE buffer (Table 2.4 ) and extracted with phenol:chloroform 3 times, with ether twice and finally precipitated with ethanol as described in Section 2.12.4. The DNA was redissolved in TE buffer.

### 2.13.3 Ligation of DNA fragments

The ligation reactions were carried out using a vector:insert molar ratio of 3:1. The ligation mix contained 2.2 $\mu$ g lambda DNA, 0.3 $\mu$ g vector DNA, 3 $\mu$ l 5mM ATP, 1 unit ligase (BRL), 3 $\mu$ l x10 ligase buffer (400mM Tris.HCl pH7.6, 100mM MgCl<sub>2</sub>, 100mM DTT) and the volume made up to 30 $\mu$ l with sterile distilled water. The ligation mix

was incubated at 15°C for 16 hours.

#### 2.13.4 Preparation of competent cells

The two *E.Coli* strains which were required to be competent for the uptake of plasmid were DS941 (for the propagation and characterisation of recombinant plasmids) and MV1190 (for DNA sequencing ). Both of these strains are described in detail in Section 2.8.3.

DS941 was streaked out onto an LB agar plate (Table 2.3) to obtain a stock plate of bacterial colonies. A single colony of DS941 was transferred to 10ml LB medium (Table 2.3) and grown overnight at 37°C. 100ml LB medium supplemented with 0.2% (w/v) glucose was inoculated with 1ml of the overnight culture and incubated at 37°C with good aeration until the optical density at 600nm was 0.3. The culture was incubated on ice for 10 minutes, then harvested at 6,000 rpm (6,000xg<sub>av</sub>) in the Sorvall GSA rotor for 5 minutes at 4°C. The medium was decanted and the bacterial pellet gently resuspended in 6ml ice-cold CaCl<sub>2</sub> in 10mM Tris.HCl pH8. The resuspended pellet was incubated on ice for 30 minutes, then centrifuged at 3,000 rpm (1,500xg<sub>av</sub>) for 5 minutes at 4°C in the Sorvall HB4 rotor. The supernatant fraction was decanted and the bacterial pellet very gently resuspended in 2ml ice-cold 100mM CaCl<sub>2</sub>. The bacterial cells were now competent and ready for transformation. Fresh competent cells were prepared for each transformation.

MV1190 bacterial cells were made competent as described for DS941 bacteria with the following exceptions: a single colony of MV1190 was picked from a stock grown on minimal agar (Table 2.3) and the colony was then propagated in 2xTY medium (Table 2.3)

### 2.13.5 Transformation of competent *E. Coli* by plasmid DNA.

3 $\mu$ l and 15 $\mu$ l of ligation mix (Section 2.13.3) was added to 100 $\mu$ l of competent cells (Section 2.13.4) and then incubated on ice for 30 minutes. The cells were heat-shocked at 42°C for 3 minutes, 1ml of LB medium (Table 2.3) was added and the cells incubated at 37°C for 45 minutes to allow the transformed bacteria to express the antibiotic resistance gene. At least 5 minutes before use, 0.5% (w/v) 5-bromo-4-chloro-3-indolyl- $\beta$  D galactoside (Xgal, BRL) in dimethyl formamide and 0.5% (w/v) isopropyl  $\beta$ -D thiogalactopyranoside (IPTG, Sigma) in water were spread over the surface of LB agar plates supplemented with 100 $\mu$ g/ml Ampicillin (Sigma). Different amounts of transformation mix (one tenth and half of the mix) were spread onto the LB/Ampicillin/Xgal/IPTG plates. When the liquid had been adsorbed, the plates were inverted and incubated at 37°C for 16 hours. After this time, positive (white) colonies were subcultured onto similar plates as above and again incubated overnight at 37°C to ensure that they were not false positives. Colonies which continued to be white after several rounds of screening were isolated and used to purify plasmid DNA (Sections 2.12.2 and 2.12.3) or to prepare single stranded DNA for use in DNA sequence analysis (Section 2.14.2)

### 2.14 SEQUENCING DNA BY THE SANGER DIDEOXY CHAIN TERMINATION METHOD

The cDNA clones, pRLRI 7 and pRLRI 8 were sequenced by the Sanger chain termination method as described in the "M13 Cloning and Sequencing Handbook" (Amersham International)

A single stranded template is required for synthesis of the



complementary strand by the Klenow fragment of *E.coli* DNA polymerase I. The Klenow fragment lacks the 5' to 3' exonuclease activity of the intact enzyme and requires a 3' OH group to synthesise in a 5' to 3' direction. A primer, i.e. a short region of double stranded DNA, is annealed to the multiple cloning region and acts to direct the synthesis of the DNA chain. Dideoxy sequence analysis depends on the random incorporation of dideoxyribonucleotides into the growing chains. As these nucleotides lack a 3' hydroxyl group, the Klenow fragment is unable to form a phosphodiester bond with another deoxyribonucleotide and hence chain termination results. To determine the sequence, four separate synthesis reactions are performed, each containing only one of the four dideoxyribonucleotides. The ratio of deoxy NTP:dideoxy NTP is controlled so that chain termination occurs randomly over the complete sequence. The DNA chains are then separated according to molecular weight by gel electrophoresis. The development of high definition, thin polyacrylamide gels has made possible the separation of DNA fragments differing in size by a single nucleotide.

#### 2.14.1 Propagation of M13KO7 Helper Phage

The M13KO7 helper phage were kindly donated by Dr L.M Anderson of this department and were propagated as follows:

Phage were streaked onto an LB agar plate (Table 2.3). 4ml of top agar (Table 2.3) containing 0.5ml of an overnight culture of MV1190 was poured across the plate and it was incubated overnight at 37°C. The agar was scraped into a 250ml flask containing 50ml 2xTY medium (Table 2.3) and 70µg/ml Kanamycin (Sigma). and grown for 10 hours

with good aeration. The bacteria were harvested at 8,000 rpm (10,000 $\times$ g<sub>av</sub>) in the Sorvall HB4 rotor and the supernatant fraction, which contained the M13KO7 helper phage, saved. The supernatant fraction was recentrifuged at 8,000 rpm to ensure that there was no contamination with bacteria. The supernatant fraction was then stored at 4°C.

#### 2.14.2 Preparation of single stranded pTZ DNA

MV1190 transformed with either pRLRI 7 or pRLRI 8 were grown at 37°C overnight in 10ml minimal medium (Table 2.3) containing 100 $\mu$ g/ml Ampicillin (Sigma). 10ml 2xTY medium (Table 2.3), also supplemented with Ampicillin, was inoculated with 100 $\mu$ l of the overnight culture and grown at 37°C until the OD at 660nm was 0.5. 2ml of this culture was transferred to a 50ml Falcon tube and inoculated with M13KO7 at a multiplicity of 10, i.e. 10 plaque forming units (pfu) M13KO7 per MV1190 cell (assuming an OD of 1 is 8 $\times$ 10<sup>8</sup> cells/ml), and incubated at 37°C for 1 hour with good aeration. 10ml 2xTY, supplemented with 70 $\mu$ g/ml Kanamycin, was inoculated with 400 $\mu$ l of infected cells and incubated at 37°C overnight with vigorous shaking. The bacteria were harvested by centrifugation at 6,000 rpm (6,000 $\times$ g<sub>av</sub>) in the Sorvall HB4 rotor and the supernatant fraction retained. The supernatant fraction, which contains the packaged pTZ DNA, was recentrifuged until there was no evidence of a bacterial pellet. The DNA was precipitated by the addition of 1ml PEG (25% (w/v) in 3M NaCl) to the supernatant fraction and the solution incubated at room temperature for 15 minutes. The precipitated phage were harvested by centrifugation at 8,000 rpm (10,000 $\times$ g<sub>av</sub>) in the

Sorvall HB4 rotor, resuspended in 350 $\mu$ l 20mM Tris.HCl pH7.5, 20mM NaCl and 1mM EDTA and transferred to an Eppendorf tube. The DNA was then phenol:chloroform extracted and precipitated as described previously (Section 2.12.4) and the DNA pellet resuspended in 50 $\mu$ l TE buffer (Table 2.3).

### 2.14.3 Annealing template to primer

The first step in the sequencing reaction was to anneal the primer to the template in order to provide a double stranded priming region for the Klenow catalysed elongation. The primer used routinely was the 15 base reverse sequencing primer which anneals 3' to the multiple cloning region (see Figure 2.2).

5 $\mu$ l (approximately 1 $\mu$ g of DNA) of single stranded DNA template (Section 2.14.2) was annealed to 1 $\mu$ l of reverse primer (1.2mg/ml) in a final volume of 10 $\mu$ l containing 1.5 $\mu$ l of 10X Klenow reaction buffer (100mM Tris.HCl pH8.0, 50mM MgCl<sub>2</sub>). The samples were placed in a boiling water bath at 90°C, the heat turned off and the samples left for 45 minutes.

### 2.14.4 Chain elongation with Klenow polymerase

The annealed primer/template prepared as described in the previous section (Section 2.14.3), was spun briefly to the bottom of the Eppendorf tube. 15 $\mu$ Ci [ $\alpha$ -<sup>35</sup>S] dATP (Amersham) and 1 unit of Klenow fragment (Amersham) were added and the contents of the tube mixed carefully by pipetting in and out.

Four Eppendorf tubes, marked A, C, G, and T, were placed in an Anderman 4515 microfuge and into each was put 2.5  $\mu$ l of the above

TABLE 2.5 COMPOSITION OF dNTP/ddNTP MIXES.

The composition of deoxy (d) NTP/dideoxy (dd) NTP mixes used in Section 2.14.4 are shown. Stock solutions were provided in the Amersham sequencing kit and the mixes were prepared as described by the manufacturer in the "M13 Cloning and Sequencing Handbook" (Amersham)

deoxy (d)/dideoxy (dd) nucleotide mixes contained the following:

d/ddA 62.5 $\mu$ M of dCTP, dGTP, and dTTP, and 50 $\mu$ M of ddATP

d/ddC 82 $\mu$ M of dGTP and dTTP, 4 $\mu$ M of dCTP, and 50 $\mu$ M of ddCTP

d/ddG 82 $\mu$ M of dCTP and dTTP, 4 $\mu$ M of dGTP, and 150 $\mu$ M of ddGTP

d/ddT 82 $\mu$ M of dCTP and dGTP, 4 $\mu$ M of dTTP, and 250 $\mu$ M ddTTP

primer/template/label/enzyme mix. 2  $\mu$ l of the relevant dNTP/ddNTP mix (Table 2.5) was added to the appropriate tube and the reactions started by a brief spin. After 20 minutes, 2  $\mu$ l of chase mixture (0.5mM of all 4 dNTPs) was added and the samples incubated for a further 15 minutes. This elongates the DNA chains not terminated by a dideoxynucleotide and therefore helps to reduce the appearance of non-specific bands on the sequencing gel. The samples were then split into two: one half for immediate electrophoresis; the other half was stored at -20°C until required. 2  $\mu$ l of formamide dye mix (0.1% (w/v) xylene cyanol FF (Sigma), 0.1%(w/v) bromophenol blue (Sigma), 20mM EDTA in deionised formamide (Fluka)) was added to the sample to be electrophoresed immediately. The sample was then heated for 2 minutes at 90°C and loaded onto a polyacrylamide gel apparatus (Shandon Vertical Slab Unit 400, Southern) for determination of the DNA sequence (see following section for details).

#### 2.14.5 Polyacrylamide gel electrophoresis in the analysis of DNA sequence

The DNA samples were separated by electrophoresis on polyacrylamide/urea denaturing gels (containing 6% acrylamide /bisacrylamide (19:1) and 7M urea) using a TBE (Table 2.3) buffering system. The gel plates were siliconised prior to use with Repelcote (dimethyldichlorosilane in 1,1,1, trichloroethane) and assembled with 0.4mm spacers. The wells for loading the samples were prepared by inserting a 20 tooth comb.

The gel was pre-run at 30mA for 15 minutes prior to sample application using an LKB 2197 power supply. The samples, prepared

as described previously (Section 2.14.4), were loaded immediately after boiling and subjected to electrophoresis for between 1 and 3 hours. Electrophoresis for 1 hour at 30mA was sufficient to resolve the first 200 bases from the primer region. Further resolution was obtained by electrophoresing the second half of the reaction mix for 3 hours.

After electrophoresis, the gel was fixed in 10% (v/v) methanol, 10% (v/v) acetic acid for 20 minutes at room temperature, then dried using a vacuum gel dryer. The gel was then exposed using Fuji RX film overnight at room temperature.

## 2.15 PREPARATION OF RNA

To prevent contamination with ribonucleases, all solutions used in the preparation of RNA were treated with diethylpyrocarbonate (DEPC, Sigma) before autoclaving (described in Section 2.2) and all glassware was treated with Repelcote as described in the same section.

### 2.15.1 Isolation of total RNA

RNA was isolated from rat tissues using a modification of the guanidine isothiocyanate method of Chirgwin *et al.*, (1979).

The tissue of interest was removed from a freshly-killed rat and placed into an ice-cold solution of saline (0.9% (w/v) NaCl) in a sterile beaker. The saline was then poured off, the tissue chopped into small pieces and transferred to a chilled mortar containing liquid N<sub>2</sub>, ground to a fine powder and then stored in a plastic universal container in liquid N<sub>2</sub> until required. 6ml of chilled GIT-mercaptoethanol buffer (4M guanidine isothiocyanate, 25mM sodium acetate pH6.0, 120mM β-mercaptoethanol) was pipetted into a glass homogeniser, 1g of

powdered tissue added and the tissue homogenised with a motor-driven pestle (approximately 10 strokes). The resultant "slurry" was drawn into a syringe and the DNA sheared by passing through a G23 needle five times. The solution was transferred to sterile Corex tubes and centrifuged at 10,000 rpm ( $16,000xg_{av}$ ) in the HB4 rotor of a Sorvall RC-5 centrifuge for 10 minutes at 4°C to remove cellular debris. 6ml of caesium chloride buffer (5.7M caesium chloride, 25mM sodium acetate pH6.0) was pipetted into ultracentrifuge tubes and the guanidine-lysed sample layered on top. The tube was filled with GIT-mercaptoethanol buffer and the sample centrifuged at 31,000 rpm ( $116,000xg_{av}$ ) in a SW.40 rotor of a Beckman LS-50 ultracentrifuge for 20 hours at 20°C. After this time, the centrifuge was stopped without the use of brakes to prevent disturbing the bands of protein and DNA. The RNA was observed as a gelatinous pellet at the bottom of the tube. The supernatant fraction was removed carefully with a sterile pasteur pipette and the tube inverted over a tissue to drain. The RNA pellet was resuspended in 1ml GIT-mercaptoethanol and transferred to a Corex tube. The ultracentrifuge tube was washed out with 2x1ml aliquots of the same buffer which was also transferred to the Corex tube. The volume was then increased to 6ml with GIT-mercaptoethanol and the RNA extracted with an equal volume of phenol:CHCl<sub>3</sub>. The phases were separated by centrifugation at 10,000rpm ( $16,000xg_{av}$ ) in the HB4 rotor of the Sorvall for 20 minutes. The upper aqueous phase was extracted with chloroform as above and the RNA precipitated as described in Section 2.15.2.

An aliquot of resuspended RNA was obtained and analysed by spectrophotometer to determine the OD 260/280 ratio (a pure sample of RNA has an OD 260/280 ratio of 2) and the quantity of RNA (an



OD260 of 1 is equivalent to 40 $\mu$ g/ml RNA). In general, 1g of rat tissue yielded approximately 1000 $\mu$ g of total RNA.

#### 2.15.2 Ethanol precipitation of RNA preparations

RNA was precipitated by estimating the volume of the sample, adding 1/10 volume of 3.3M Na acetate pH5.5 and 2.5 volumes of absolute alcohol (Burrough) and incubating overnight at -20°C.

In general, the RNA was stored as an ethanol precipitate at -20°C until required. When needed, the RNA was pelleted by centrifugation at 10,000 rpm (16,000 $\times$  $g_{av}$ ) in the Sorvall HB4 rotor at 4°C. The pellet was washed with 70% (v/v) absolute alcohol, dried under vacuum and resuspended in an appropriate volume of DEPC-treated double deionised distilled water ("super Q" water).

### 2.16 PREPARATION OF PROBES FOR USE IN NORTHERN AND SOUTHERN BLOTTING

#### 2.16.1 Purification of cDNA insert

To isolate the required cDNA insert, 5 $\mu$ g of recombinant plasmid (pRLRI 7, pRLRI 8 or pHPI) was digested with EcoRI as described in Section 2.13.1 and the digested products subjected to electrophoresis on an 1% low melting point agarose gel (Section 2.9.7) at 40V until the tracking dye neared the end of the gel. The gel was illuminated using a UV transilluminator and the DNA band corresponding to the cDNA insert was excised, diced into small pieces and transferred to a sterile 1.5ml Eppendorf tube. The tube containing the gel was heated to 65°C for 5 minutes to melt the agarose and the volume made to 200 $\mu$ l with

TE buffer (Table 2.4). A further 2 volumes of TE buffer was added and the cDNA insert was extracted with 1 volume of TE-saturated phenol (the phenol was pre-equilibrated to 65°C). The resultant aqueous phase was extracted a further twice with phenol, once with phenol:chloroform at room temperature and once with chloroform. The cDNA was precipitated as described previously (Section 2.12.4). The resultant pellet was resuspended in 10µl TE buffer and stored at -20°C until required. In general, it was assumed that, from 5µg of recombinant plasmid, 250ng of pRLRI 7 and 8 insert and 800ng of pHPI insert was obtained (assuming the inserts from pRLRI 7 and 8 were one tenth of the total DNA, the insert from pHPI was one third of total DNA and 50% of the DNA was lost during phenol extraction and subsequent stages).

#### 2.16.2 Labelling of cDNA inserts

The cDNA inserts were labelled by the random priming method of Feinberg and Vogelstein (1983). Denatured DNA acts as a template for the Klenow fragment of DNA polymerase using random oligonucleotides as primers.

50ng of insert cDNA, purified as described in the previous section (Section 2.16.1), was diluted to 35µl with TE buffer (Table 2.3) in a sterile Eppendorf tube, incubated for 5 minutes in a boiling water bath to denature the DNA and then plunged into ice for 2 minutes to prevent the denatured DNA from reannealing. 10µl of hexanucleotide solution (Table 2.6) and 1µl of BSA (20mg/ml, Boehringer Mannheim) were added and the contents of the tube spun briefly. 30µCi [ $a^{32}P$ ] dCTP (Amersham) was added, followed by 3 units of Klenow fragment

10

TABLE 2.6 COMPOSITION OF THE RANDOM PRIMER SOLUTION

The hexanucleotide random primer solution used in Section 2.16.2 is outlined. The solution was prepared as described, aliquoted into 50 $\mu$ l lots and stored at -20°C.

Solution A: 0.5mM deoxytriphosphates (dATP, dGTP, dTTP) in 1.25M Tris.HCl pH8, 0.125M Mg Cl<sub>2</sub>, 10mM β-mercaptoethanol

Solution B: 2M HEPES pH6.6

Solution C: 50 units hexadeoxynucleotides (Pharmacia) resuspended in 556μl distilled water.

The hexanucleotide solution was prepared by mixing together solutions A, B and C in the ratio 100:250:150.

(Boehringer Mannheim). The reaction mix was then incubated at room temperature for approximately 16 hours. To stop the reaction, 2 $\mu$ l 0.5M EDTA and 2 $\mu$ l 20% (w/v) SDS were added to the solution. At this point 1 $\mu$ l of the reaction mix was diluted to 50 $\mu$ l with TE buffer and processed as described in the following paragraph. The remainder of the labelled insert was precipitated as described in Section 2.12.4 and resuspended in 10 $\mu$ l TE buffer.

The amount of radioactivity incorporated into the cDNA was determined by spotting 5 $\mu$ l aliquots onto two separate Whatman DE 81 filters that were allowed to dry. One filter (A) was placed directly into a scintillation vial and 5ml Ecoscint added. The other filter (B) was washed with 0.5M Na<sub>2</sub>HPO<sub>4</sub> for 30 minutes with 6 changes of buffer, then several times with distilled water and finally with absolute alcohol. These washes remove any label which has not been incorporated into the cDNA. The filter was allowed to dry and added to a scintillation vial with 5ml Ecoscint. The two filters were then counted on an LKB 1209 Rackbeta scintillation counter. Filter A is a measure of the total counts obtained whereas filter B is a measure of the label which has been incorporated into the cDNA insert. In general, the random priming method of labelling the cDNA inserts gave incorporation of between 70 and 85%.

## 2.17 NUCLEIC ACID HYBRIDISATION

### 2.17.1 Northern blotting

This technique involves the separation of RNA by electrophoresis on an agarose gel under denaturing conditions, transfer of the RNA onto a solid support i.e. a membrane filter, and then detection of specific

RNA species by hybridisation with a radioactive probe.

Samples of RNA were denatured by heating them to 65°C for 5 minutes in 20mM MOPS (3-[N]-morpholinopropane sulphonic acid, Sigma), 50% (v/v) deionised formamide and 2.2M formaldehyde. They were then rapidly cooled on ice and made 0.04% (w/v) with respect to bromophenol blue (Sigma).

A 1% agarose gel containing 2.2M formaldehyde was prepared and the RNA sample (prepared as described in the above paragraph) applied to the gel. Electrophoresis of the RNA samples was carried out overnight at 25V using 20mM MOPS as the buffering system.

After electrophoresis, the RNA was transferred from the agarose gel to a nylon membrane (Hybond N, Amersham) as described by Thomas (1980). Whatman 3MM paper, saturated with 20X SSC (see Table 2.3), was laid over a glass plate such that two of its edges were dipping into reservoirs of 20X SSC. The agarose gel was placed on the saturated 3MM paper and cling film placed around all four edges. Hybond N was laid carefully over the gel to ensure that no air was trapped between the gel and filter. Three pieces of dry 3MM paper were then placed on top of the gel, followed by a 6cm thickness of paper towels and a glass plate. A heavy weight was then placed on top of this and the assembly left for 4 hours with the wet paper towels being replaced every 15 minutes. After this time, the membrane filter was dried at room temperature, wrapped in Saranwrap and the RNA cross-linked to the filter by exposure to UV light for 1 minute.

The membrane filter was prehybridised by incubating at 42°C with 10ml buffer 1 (30% (v/v) deionised formamide (Fluka), 5X Denhardt's (Table 2.3), 5X SSPE (Table 2.3), 0.1% (w/v) SDS and 100µg/ml denatured salmon sperm DNA) for 4 hours in a perspex, water-tight

box. Single stranded radioactive probe (labelled by the random priming method as described in Section 2.16.2) was added to 10 ml of buffer 1 at a concentration of  $10^6$  cpm/ml and incubated with the Hybond N filter at  $42^\circ\text{C}$  for 16 hours. The filter was then removed from the hybridisation chamber into a plastic sandwich box and rinsed with 2X SSPE containing 0.1% (w/v) SDS at  $42^\circ\text{C}$ . Non specifically bound probe was removed by incubating the filter with 1X SSC containing 0.1% (w/v) SDS for 15 minutes at  $65^\circ\text{C}$  (medium stringency wash). The filter was then washed with high stringency (0.1X SSC, 0.1% (w/v) SDS) for 15 minutes at  $65^\circ\text{C}$ . The hybridisation of probe to blotted RNA was visualised by autoradiography i.e. the filter was exposed to Kodak-X-Omat film using an intensifying screen (Cronex lighting) and left for an appropriate time period at  $-70^\circ\text{C}$ . It should be noted that when a non homologous probe was used in the northern blot technique, the filter was exposed after the medium stringency wash, then subjected to a high stringency wash and re-exposed.

The radioactive probe may be removed from the northern blot by incubating the membrane in a solution containing 5mM Tris.HCl pH8.0, 2mM EDTA and 0.1xDenhardt's (Table 2.4) for 2 hours at  $65^\circ\text{C}$ . The blot may then be challenged with an alternative probe.

### 2.17.2 Determination of the size of RNA transcripts

An RNA ladder (Boehringer Mannheim) was electrophoresed in the last track of all RNA gels and blotted with the other RNA samples onto Hybond-N as described in Section 2.17.1. After cross-linking, the section of filter containing the RNA ladder was removed, stained with methylene blue for 5 minutes and destained in  $\text{H}_2\text{O}$  until the RNA bands were visible (Section 2.17.3). Rf values were then calculated i.e:

$$R_f = \frac{\text{distance migrated by RNA standard}}{\text{distance migrated by tracking dye}}$$

A graph of log bp versus  $R_f$  values was plotted using the standards from the RNA ladder. The size of specific transcripts present on the blot were then calculated using the standard curve.

Similarly, a 123 bp ladder was used to determine the size of DNA fragments on an agarose gel. The DNA bands were visualised by direct ethidium bromide staining of the agarose gels (as described in Section 2.9.7)

### 2.17.3 Staining of RNA markers

RNA markers were visualised following their transfer onto Hybond N as recommended by Amersham. The membrane containing the markers was incubated at room temperature in 5% (v/v) acetic acid for 15 minutes and then stained in a solution containing 0.5M sodium acetate, pH5.5, 0.04% (w/v) Methylene Blue (Sigma) for 5 minutes. The stain was removed by washing in distilled water. The filter was then dried and stored in the dark.

### 2.17.4 Southern blotting

The principle of Southern blotting is very similar to that of northern blotting except that DNA is separated by electrophoresis under non-denaturing conditions (Southern, 1975)

The DNA was subjected to electrophoresis on a 1% (w/v) agarose gel at 50V for approximately 3 hours using 1X TBE (Table 2.3) as the



buffering system. The DNA was denatured by incubating with 0.5M NaOH, 1.5M NaCl for 30 minutes at room temperature and then neutralised by soaking in 0.1M Tris.HCl, pH7.5, 1.5M NaCl for a further 30 minutes. The DNA was transferred onto Hybond N essentially as described for the transfer of RNA in the previous section (Section 2.17.1), with the exception that the transfer took place overnight. The filter was removed after this time and soaked in 2X SSC (Table 2.3) for 10 minutes, dried between two pieces of 3MM paper, then baked for 2 hours at 80°C in a vacuum oven.

The filter was incubated in 10ml prehybridisation buffer (5X Denhardt's (Table 2.3), 6xSSC (Table 2.3), 0.1% (w/v) SDS, 100µg/ml salmon sperm DNA) for 4 hours at 65°C and then hybridised overnight at 65°C in fresh buffer containing  $1 \times 10^6$  cpm/ml of labelled probe (Section 2.16.2). The filter was washed at 65°C for 3x30 minutes in 2X SSC, 0.1% (w/v) SDS and finally in 0.2X SSC, 0.1% (w/v) SDS for 30 minutes. The membrane filter was then exposed to Kodak X-Omat film using an intensifier screen (Cronex lighting) and left for an appropriate time period at -70°C.

The control probe used in both northern and Southern blotting was kindly donated by D.P.Leader of this department and was a 700bp mouse  $\gamma$  actin cDNA in the xba/pst 1 sites of pUC 18.

**CHAPTER THREE**

**RESULTS**

This chapter is divided into several sections: the first illustrates the preparation of the antibody probe which was used to screen the rat liver  $\lambda$ gt11 expression library; the second section outlines the isolation of the cDNA clones from this library and the final sections describe the characterisation of these clones by immunological methods and by direct comparison of amino acid and nucleotide sequences with those of ribonuclease inhibitor purified from human placenta (Lee *et al.*, 1988) and porcine liver (Hofsteenge *et al.*, 1988).

### **3.1 PREPARATION OF THE ANTIBODY PROBE**

#### **3.1.1 Purification of ribonuclease inhibitor from rat liver**

Ribonuclease inhibitor was routinely prepared by the method of Burton (1980) using rat liver as the source of protein. Details of the purification procedure are outlined in Sections 2.5.1 and 2.5.2. Initially, ribonuclease inhibitor was purified to be used as an antigen in the production of antiserum (see Sections 2.7.1 and 3.1.2 for details). In the later stages of the project, the protein was purified for use in amino acid sequence analysis (outlined in Section 3.4).

A purification table for a typical preparation is shown (Table 3.1) and is comprised of the data obtained from a preparation utilising 20 rat livers and a 1ml sepharose affinity column. The first step, the preparation of a 35-60% (w/v) ammonium sulphate

**TABLE 3.1 PURIFICATION OF RAT LIVER RIBONUCLEASE****INHIBITOR**

The data was derived from a typical purification using the livers of 20 Wistar rats and a 1ml RNase A-Sepharose affinity column (described in Section 2.5). Inhibitor activity was assayed as described in Section 2.5.4. Protein concentration was determined by the method of Bradford (Section 2.4.1).

1 unit of inhibitor activity was defined as the quantity required to inhibit by 50% the digestion of 5ng of RNase A.

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification factor	% Yield
Rat liver cytoplasm	14,000	186,000	13.3	0	100
35-36% ammonium sulphate precipitate (after dialysis)	3,200	160,000	50	3.75	86
Affinity chromatography eluate	0.72	80,000	111,100	8,400	43

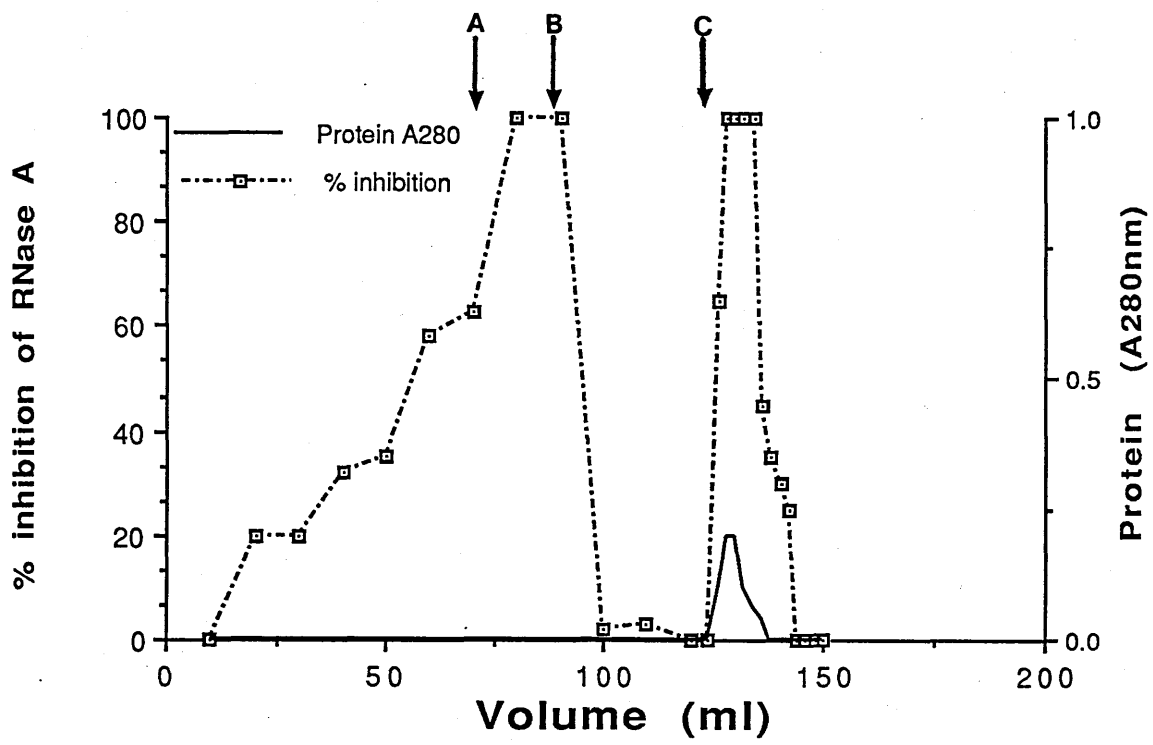
37

**FIGURE 3.1 RNASE A-SEPHAROSE AFFINITY**  
**CHROMATOGRAPHY OF RAT LIVER RIBONUCLEASE INHIBITOR**

The purification of ribonuclease inhibitor from rat liver was performed as described in Section 2.5.2. The activity of the inhibitor was determined as described in Section 2.5.4 and protein concentration was determined by absorbance at 280nm.

The starting buffer at A was 45mM potassium phosphate, pH6.4 and at B was changed to starting buffer made 0.5M with respect to NaCl. The elution buffer at C was 0.05M borate pH6; 4M NaCl; 15% (v/v) glycerol. All buffers also contained 1mM EDTA and 10mM  $\beta$ -mercaptoethanol.

The amount of protein in the fractions coming through the column prior to the addition of buffer B had an absorbance at 280nm greater than 1.0. After changing to buffer B, the absorbance at 280nm decreased until a value of 0.05 at 280nm was obtained (this data is not shown on this figure). The protein eluted from the affinity column after addition of buffer C is shown.



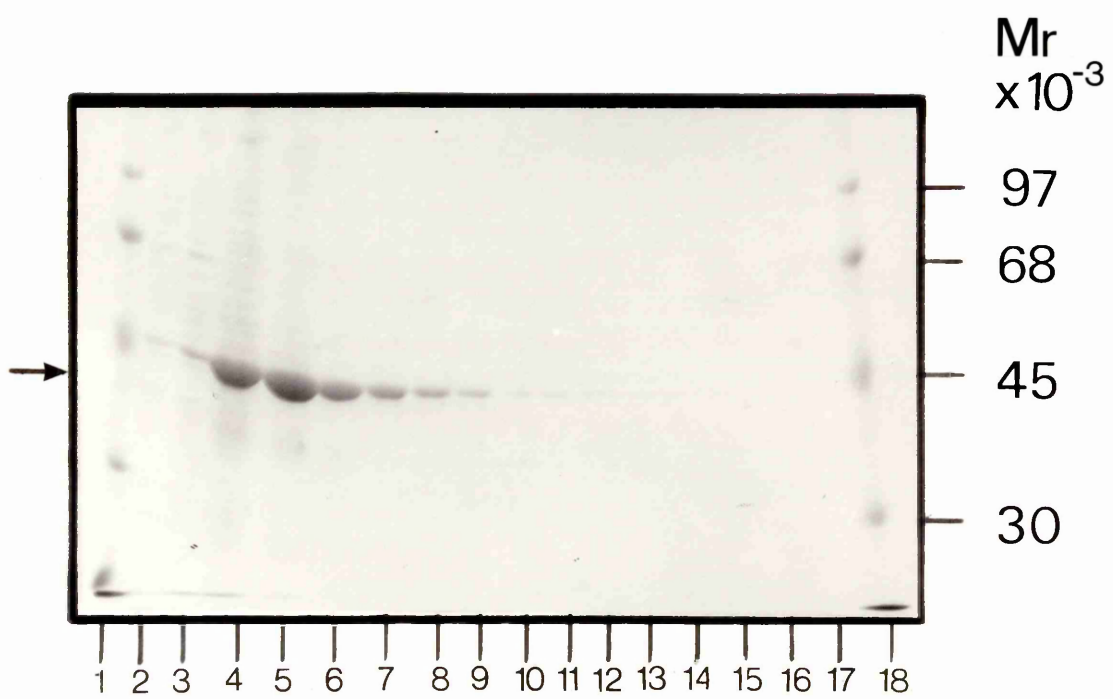
**FIGURE 3.2 SDS PAGE ANALYSIS OF FRACTIONS ELUTED  
FROM THE RNASE A-SEPHAROSE AFFINITY COLUMN**

After changing to elution buffer (0.05M borate pH6; 4M NaCl; 15% (v/v) glycerol; 10mM  $\beta$ -mercaptoethanol; 1mM EDTA), 100 $\mu$ l of each fraction eluted from the column was acetone precipitated (Section 2.4.2), the precipitate resuspended in laemmli sample buffer and subjected to electrophoresis on a 10% SDS polyacrylamide gel (Section 2.4.3). The gel was stained with Coomassie Blue and destained as described in Section 2.4.5.A.

Lanes 1 and 18 contained molecular weight markers (Sigma) as follows: myosin (200 KDa); phosphorylase b (97 KDa); bovine serum albumin (68 KDa); ovalbumin (45 KDa); carbonic anhydrase (30 KDa).

The arrow shows the position of the inhibitor.





precipitate from rat liver cytoplasm, resulted in a purification factor of approximately 4. The second step, affinity chromatography using the RNase A-Sepharose column, resulted in an overall purification of approximately 8,500 fold. The overall yield of a typical purification was approximately 40%. The run-through fraction from this stage of the purification i.e. the proteins which did not bind to the affinity column, was retained and used to remove contaminating antibodies from the immune serum (Section 2.7.6 and Section 3.2).

The profile of a typical affinity chromatography fractionation is shown in Figure 3.1. One tenth of each fraction eluted from the column after addition of the borate buffer was subjected to SDS polyacrylamide gel electrophoresis. This revealed that a major band corresponding to a protein of relative molecular mass ( $M_r$ ) of 46,700 Daltons was eluted. Several contaminating proteins were also observed (Figure 3.2).

### 3.1.2 Preparation and characterisation of immune serum raised against rat liver ribonuclease inhibitor.

Antiserum was required as a probe to screen the rat liver cDNA library in the expression vector  $\lambda$ gt11. As mentioned in Section 2.8.1, the library was constructed by insertion of foreign cDNA into the *lac Z* gene of  $\lambda$ gt11. If the cDNA of interest is in the correct frame and orientation, it may be expressed as a fusion protein with  $\beta$ -galactosidase and, therefore, an antibody can be used to screen the library by detecting the protein encoded by the cDNA of interest.

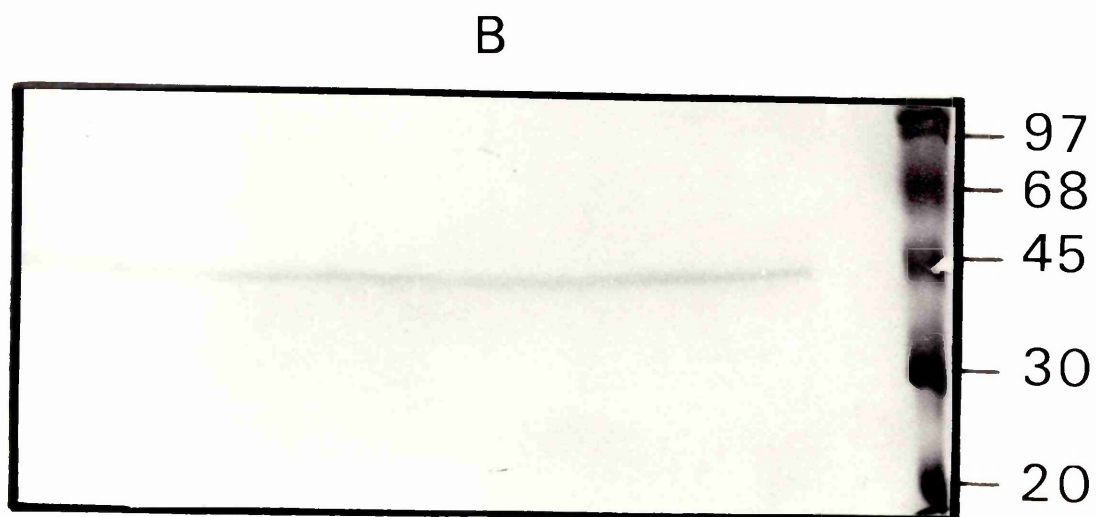
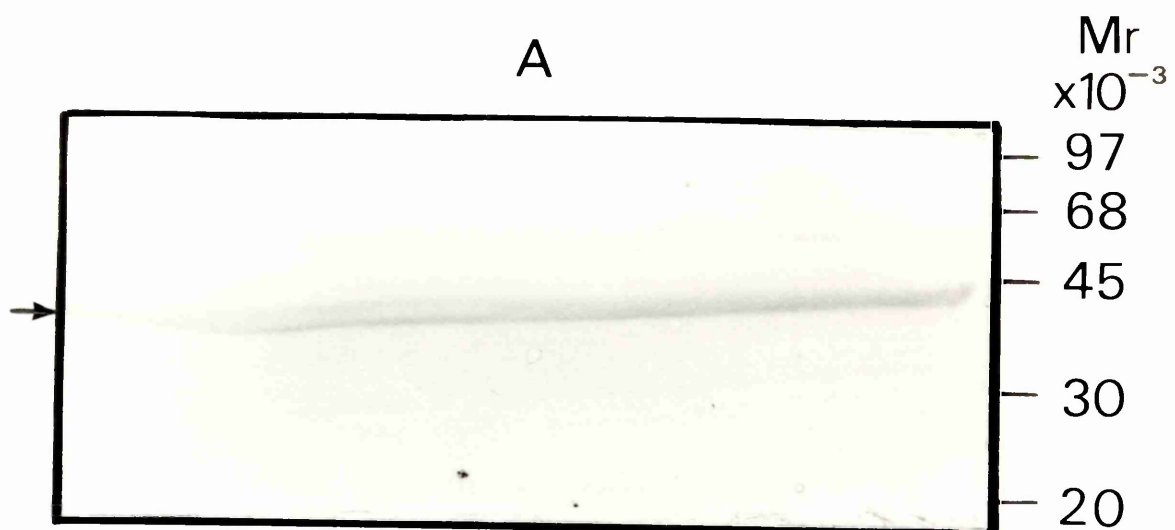
Ribonuclease inhibitor purified as described in the previous section (3.1.1), was used to produce antiserum as described in Section 2.7.1. The ribonuclease inhibitor purified by affinity chromatography contained minor amounts of contaminating proteins (Figure 3.2) and to prevent raising antibodies against these contaminants, the preparation was further purified by preparative SDS PAGE. Figure 3.3.A shows 250 $\mu$ g of inhibitor preparation after preparative electrophoresis. The band corresponding to ribonuclease inhibitor (shown by arrow) was excised and again subjected to preparative SDS PAGE to ensure that the contaminants had been removed (Figure 3.3.B). The protein band corresponding to the inhibitor after two rounds of electrophoresis was excised and used for immunisation.

The titre of the immune serum was determined by ELISA (Section 2.7.4) and was defined as the dilution required for immune serum to give the same reading at 492nm as did pre-

**FIGURE 3.3 PREPARATIVE SDS-PAGE OF RIBONUCLEASE  
INHIBITOR.**

250 $\mu$ g ribonuclease inhibitor, purified by affinity chromatography as described in Section 2.5.2., was subjected to SDS PAGE on a preparative 10% (w/v) polyacrylamide gel (Section 2.4.6), then stained with Coomassie Blue and destained as described in Section 2.5.4.A. The band corresponding to ribonuclease inhibitor was excised (shown by arrow in Figure A) and re-electrophoresed (Figure B).

The molecular weight markers are described in Section 2.4.8.



immune serum. Two rabbits were immunised, one of which gave antiserum with a workable titre (1/100,000). This titre was obtained after 5 booster injections following the initial injection. This rabbit provided antiserum for the duration of the project.

One method of ascertaining whether the immune serum has been raised against the protein of interest is to determine if the activity of that protein is decreased in the presence of the antibody. Increasing amounts of immune serum were added to a ribonuclease inhibitor assay. The assay contained a fixed amount of inhibitor which reduced the activity of 0.25ng RNase A by 100%. The fixed amount of inhibitor was determined by titrating 0.25ng of RNase A with increasing amounts of ribonuclease inhibitor (Figure 3.4). Extrapolation of the curve revealed that 27ng of inhibitor totally inactivated 0.25ng of RNase A.

Thus, increasing amounts of immune serum were added to an inhibitor assay containing 27ng of inhibitor and 0.25ng of RNase A. The activity of the inhibitor was observed to decrease as increasing amounts of immune serum were added. After the addition of 8 $\mu$ l immune serum, the inhibitor was fully inactivated (Figure 3.5). As a control, increasing amounts of pre-immune serum i.e. serum taken from the rabbit prior to immunisation, were added to the inhibitor assay. This revealed that the addition of 16 $\mu$ l of pre-immune serum also decreased the activity of the inhibitor. However, less immune serum was required to fully inactivate ribonuclease inhibitor, presumably due to the presence of antibodies raised against the inhibitor.

The specificity of the antiserum was tested by western blotting

**FIGURE 3.4 TITRATION OF RNASE A WITH RIBONUCLEASE  
INHIBITOR**

Different amounts of affinity purified ribonuclease inhibitor (Section 2.5) were added to a ribonuclease inhibitor assay containing 0.25ng RNase A (Section 2.5.4).

RNase A was assumed to be 100% active in the absence of inhibitor.

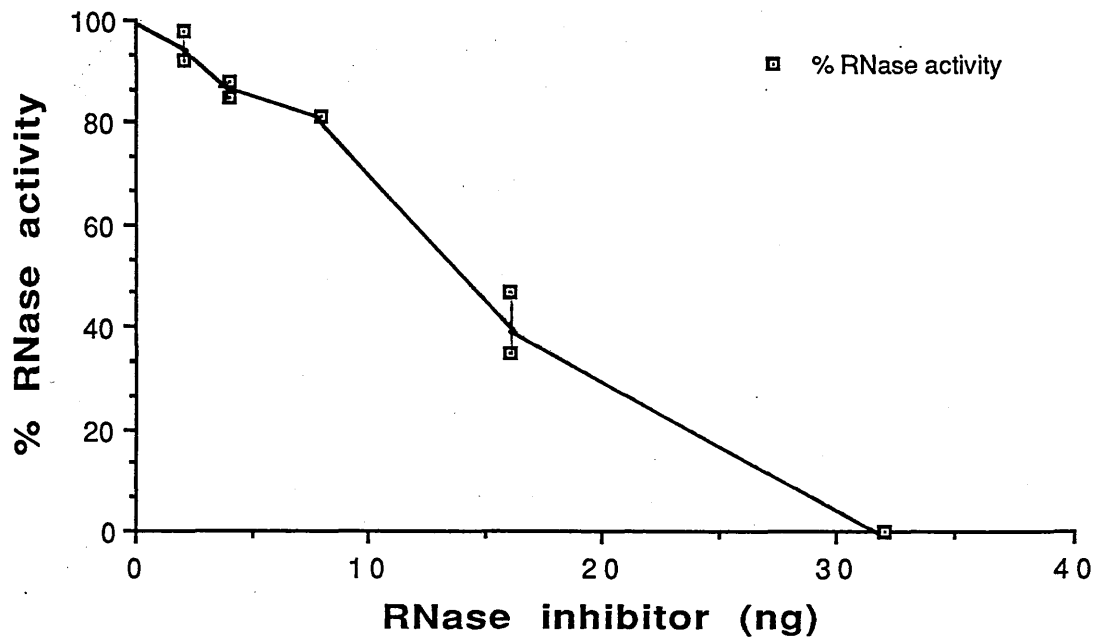
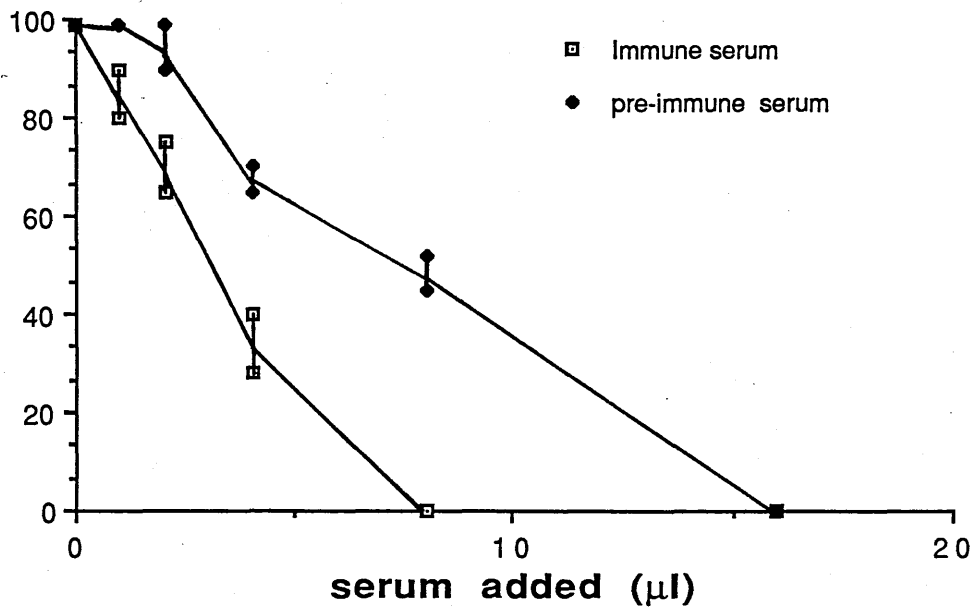




FIGURE 3.5 TITRATION OF RIBONUCLEASE INHIBITOR WITH  
PRE-IMMUNE AND IMMUNE SERUM

Varying amounts of pre-immune and immune serum were added to a ribonuclease inhibitor assay containing 27ng of inhibitor and 0.25ng RNase A. The inhibitor was assumed to be 100% active in the absence of serum.

**% activity Rnase inhibitor**



as outlined in Section 2.7.3 and the result is shown in Figure 3.6.A. The immune serum detected purified ribonuclease inhibitor (lane 3). Analysis of lane 1 shows that the immune serum detected ribonuclease inhibitor from a preparation of rat liver cytoplasm. Also present in lane 1 were several other bands of protein which did not correspond to the inhibitor. This indicated that the immune serum also contained several contaminating antibodies. It should be noted that when a similar western blot was challenged with pre-immune serum, a band corresponding to the ribonuclease inhibitor was not obtained but the additional bands were detected (data not shown). Thus, the presence of the contaminating antibodies was not a consequence of immunisation.

As the immune serum was required to probe an expression library for the isolation of ribonuclease inhibitor clones, it was necessary to remove the contaminating antibodies otherwise false positives might be obtained. This was achieved by immunoadsorption (Section 2.7.5). The run-through fraction from the RNase A-Sepharose 4B column was retained (Section 3.1) and shown to be depleted of all ribonuclease inhibitor activity by assay (Section 2.5.4) and by western blotting (Figure 3.6.A, lane 2). The western blot showed that the fraction contained little or no ribonuclease inhibitor but did contain the antigens that were recognised by the contaminating antibodies. Thus, it appeared that the run-through fraction might provide a means to remove contaminating antibodies from the antisera.

Proteins from the run-through fraction of the RNase A-Sepharose affinity column were conjugated to CNBr-Sepharose 4B and immune serum was passed through a bed of this resin (Section

**FIGURE 3.6 ANALYSIS OF IMMUNE SERUM BY WESTERN BLOTTING.**

Samples were electrophoresed, transferred to nitrocellulose and challenged with immune serum as described in Sections 2.4.3 and 2.7.3. The blots were developed using horseradish peroxidase conjugated secondary antibody (SAPU).

**FIGURE A:** This filter was challenged with pre-adsorbed immune serum.

lane 1: 100 $\mu$ g rat liver cytoplasmic proteins.

lane 2: 100 $\mu$ g run-through fraction from RNase A-Sepharose affinity column.

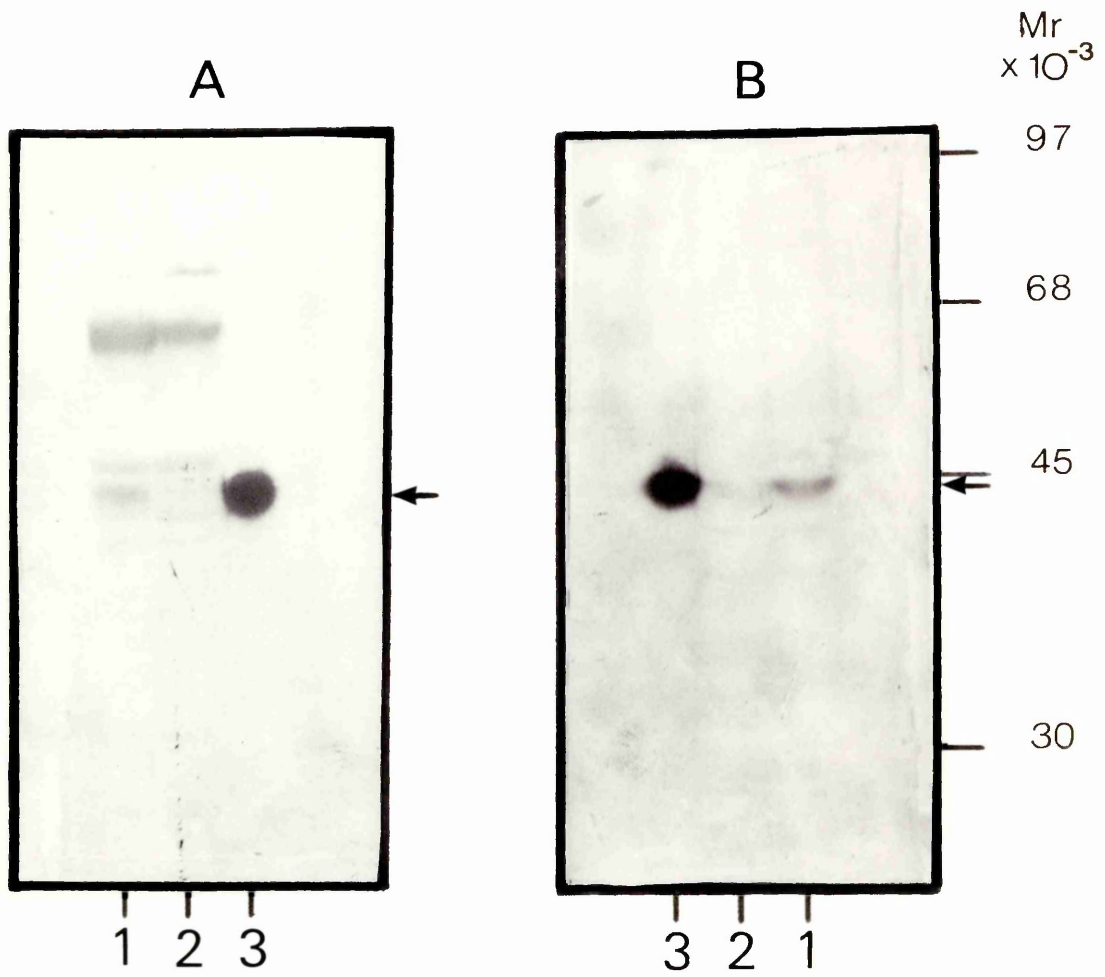
lane 3: 500ng affinity purified ribonuclease inhibitor.

**FIGURE B:** This filter was challenged with adsorbed immune serum.

Lanes 1, 2 and 3 as above.

The position of ribonuclease inhibitor is shown by the arrows.

The molecular weight markers are as described in Section 2.4.8



**FIGURE 3.7 ELISA OF FRACTIONS FROM THE**  
**IMMUNOADSORBENT COLUMN**

ELISA was performed as described in Section 2.7.4.

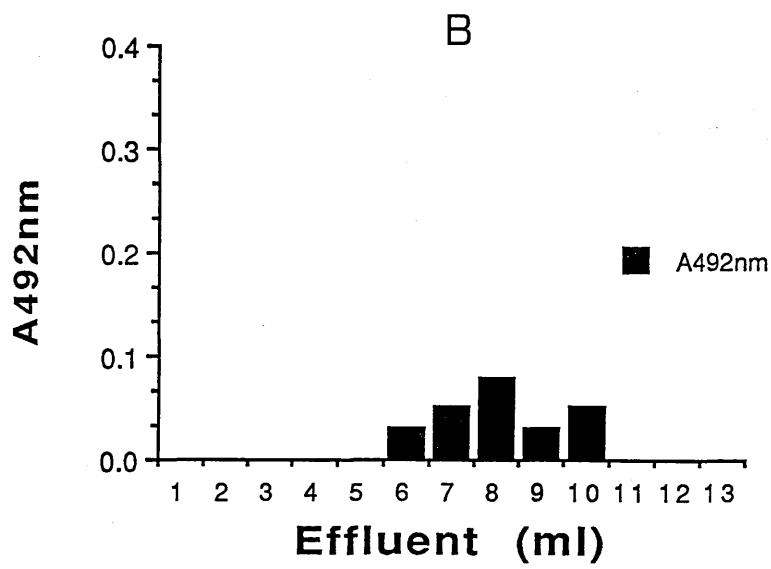
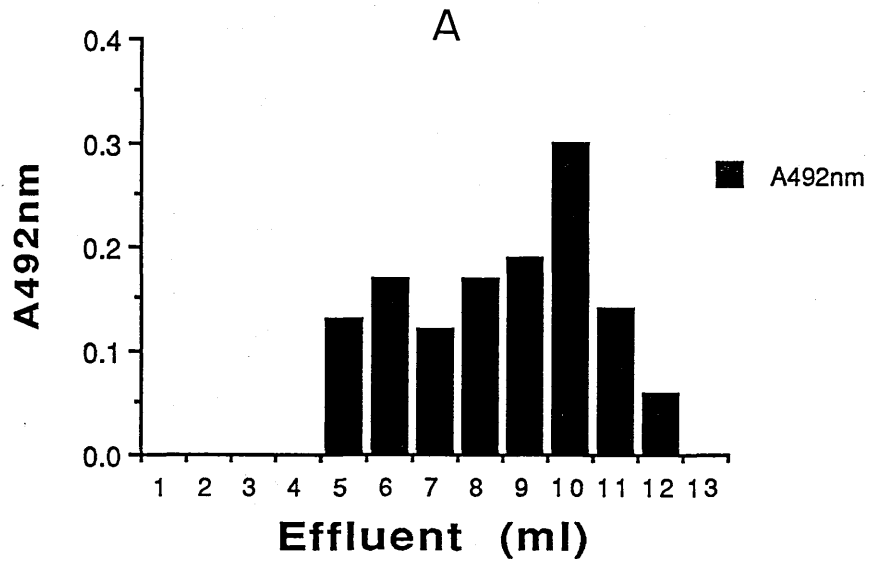
In (A), the antigen was 100ng of affinity purified ribonuclease inhibitor.

In (B), the antigen was the run-through fraction from the RNase A-sepharose affinity column diluted 1/1000 with PBS.

The antisera were the fractions from the immunoadsorbent column diluted 1/1000 in PBS.

The absorbance values were derived from the average of duplicate samples.

Immunoabsorbance was performed as described in Section 2.7.5



2.7.5). In theory, the antibodies raised against ribonuclease inhibitor should pass directly through the column as there should be little or no inhibitor conjugated to the sepharose, whereas the contaminating antibodies should bind to their respective antigens. The run-through from the immunoabsorption column was collected in 1ml fractions and tested for specificity by ELISA (Section 2.7.4). The results are shown in Figures 3.7.A and 3.7.B. In Figure 3.7.A, the antigen was purified ribonuclease inhibitor and thus fractions containing antibodies raised against inhibitor were determined. In Figure 3.7.B, the antigen was the run-through fraction from the RNase A-Sepharose affinity column. These results show that fractions 5-12 contained antibodies which recognised the ribonuclease inhibitor but did not contain appreciable amounts of antibodies which recognised other liver proteins (pre-adsorbed immune serum gave a reading of 0.5 at 495nm when tested by ELISA against the run-through fraction).

The specificity of the adsorbed immune serum was confirmed by western blotting (Figure 3.6.B). The adsorbed immune serum detected a single protein from a preparation of rat liver cytoplasm (lane 3) and this protein appeared to correspond to the inhibitor protein (lane 1). Thus, the contaminating antibodies were removed and the immune serum was now functionally monospecific i.e. it recognised only one protein.



### 3.1.3 Removal of *E.coli* antibodies from immune serum

Antibodies are present in polyclonal antiserum which bind to antigens produced by *E.coli*. This binding activity may result in a high background when screening a recombinant bacterial library and this may make the identification of positive clones difficult. However, these contaminating antibodies can be removed from serum by the method outlined in Section 2.7.6. Western blot analysis was utilised to show that the contaminating antibodies had been removed from the serum (Figure 3.8). Several preparations of cell lysates of the bacterium *E.coli* Y1089 (Section 2.8.3) were subjected to electrophoresis on a 7.5% SDS polyacrylamide gel and the proteins blotted onto nitrocellulose as described in Sections 2.4.3 and 2.7.3. The membrane in Figure 3.8.A was challenged with pre-immune serum and several bands were observed on the blot. Thus, pre-immune serum contains antibodies which recognise and bind to several proteins which are present in a bacterial cell lysate. The contaminating antibodies were removed from the serum and an identical western blot was challenged with the antiserum (Figure 3.8.B). This western blot shows that most of the contaminating antibodies have been removed from the serum. Pre-immune serum was used to show that the presence of the bands on the western blot was not due to antibodies raised against ribonuclease inhibitor cross-reacting non specifically with bacterial proteins. When immune serum was used to challenge the same western blot as shown in Figure 3.8, the same bands were present and were thus not a consequence of immunisation. The *E.coli*

**FIGURE 3.8 REMOVAL OF *E.COLI* ANTIBODIES FROM IMMUNE SERUM**

**FIGURE A:** Western blot challenged with pre-immune serum before removal of *E.coli* antibodies.

Lane 1: 100µg proteins from a crude cell lysate of IPTG-induced non-recombinant  $\lambda$ gt11/Y1089 lysogen (prepared as in Section 2.11.2).

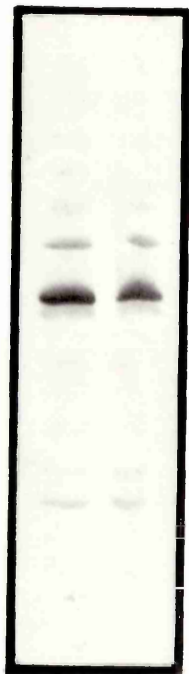
Lane 2: 100µg of non-induced crude cell lysate of non-recombinant  $\lambda$ gt11/Y1089 lysogen.

**FIGURE B:** Western blot challenged with pre-immune serum from which the contaminating *E.coli* antibodies have been removed. Lanes 1 and 2 as Figure A.

The western blot was performed as described in Section 2.7.3 and developed using the biotin-streptavidin second antibody system.

The *E.coli* antibodies were removed from the serum as described in Section 2.7.6.

A



1

2

B



1

2

antibodies were removed from the immune serum before the library was screened.

### 3.1.4 Purification of IgG from immune serum

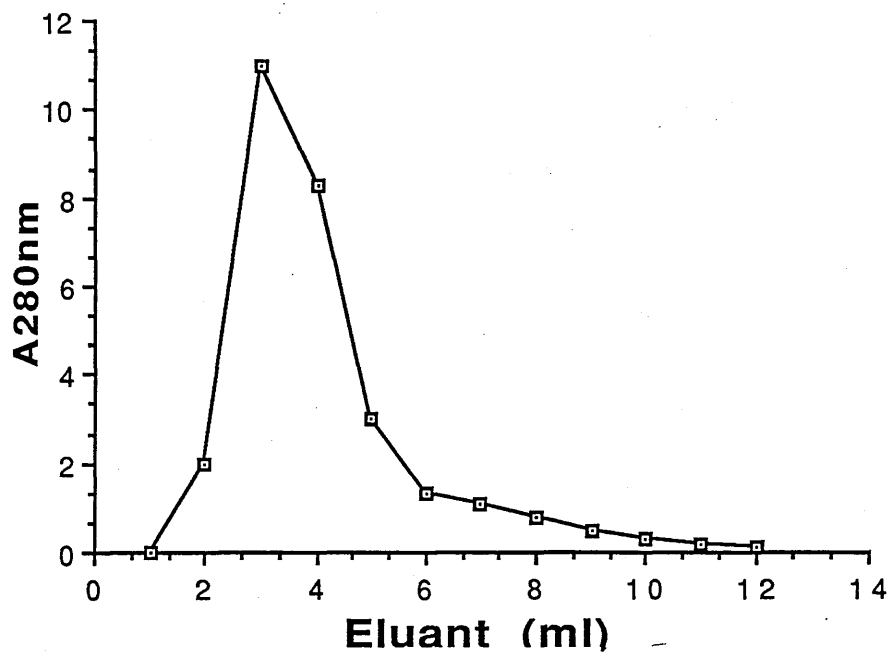
To further reduce the background when screening the library, IgG was used as antibody probe in preference to total immune serum. IgG was prepared from immune serum using the method of Johnstone and Thorpe (1982) as described in Section 2.7.2

20ml immune serum was precipitated with sodium sulphate and the resultant 14% precipitate was resuspended, dialysed and applied to a 10ml DE52 column. IgG does not bind to this anion exchange column and hence runs directly through the column. The effluent was collected in 1ml fractions and the absorbance at 280nm measured (Figure 3.9). The earliest fractions are reported to contain the purest IgG (Johnstone and Thorpe, 1982) and thus fractions 2, 3 and 4 were pooled used to screen the library.

**FIGURE 3.9 PURIFICATION OF IgG FROM IMMUNE SERUM.**

The protein concentration of 1ml fractions of the effluent from the DE.52 anion-exchange column was determined by absorbance at 280nm.

The method for purifying IgG from immune serum is described in Section 2.7.2.



### 3.1.5 Dot blot analysis of ribonuclease inhibitor with IgG

Dot blot analysis was used to determine the most sensitive method of detecting the complex formed between ribonuclease inhibitor and its antibody and also to determine the concentration of IgG with which to screen the library.

Various amounts of inhibitor (10ng, 1.0ng and 0.1ng) were dotted onto strips of nitrocellulose paper and then challenged with IgG (at a concentration of 100µg/ml). The bound antibody was detected using one of the following methods (as described in Section 2.7.3): <sup>125</sup>I-labelled protein A; horseradish peroxidase (HRP) conjugated to anti-rabbit IgG or biotinylated anti-rabbit IgG followed by streptavidin conjugated HRP. The results are shown in Figure 3.10. Detection by <sup>125</sup>I-labelled protein A appeared to be the least sensitive method as it only detected 10ng of inhibitor after an exposure time of 48 hours (Figure 3.8.A). The sensitivity of both the HRP conjugated second antibody (Figure 3.10.B) and the biotin/streptavidin system (Figure 3.10.C) appeared similar as they detected 1.0ng of ribonuclease inhibitor. However, the signal for the HRP anti rabbit IgG second antibody appeared to be slightly stronger than that for the biotin/streptavidin system. Thus it was decided to use the HRP conjugated second antibody system to detect the bound antibody in the screening of the library.

Dot blot analysis also revealed that the lowest concentration of IgG to detect 1.0ng of ribonuclease inhibitor was 50µg/ml. Thus, this concentration was used to screen the library.



**FIGURE 3.10 DOT BLOT ANALYSIS.**

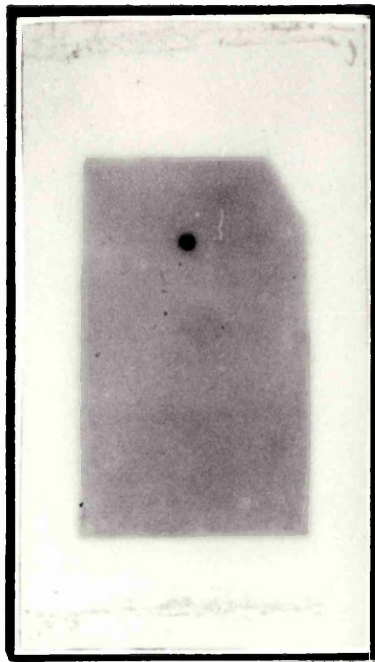
10ng, 1ng and 0.1ng of purified rat liver ribonuclease inhibitor was spotted onto strips of nitrocellulose. The control was the buffer in which the inhibitor was diluted (50mM Tris.HCl pH7.5 containing 100µg/ml BSA). The filters were challenged with IgG and bound IgG detected by one of the following methods:

**FIGURE A:**  $^{125}\text{I}$ -labelled protein A;

**FIGURE B:** horseradish peroxidase secondary antibody (SAPU)

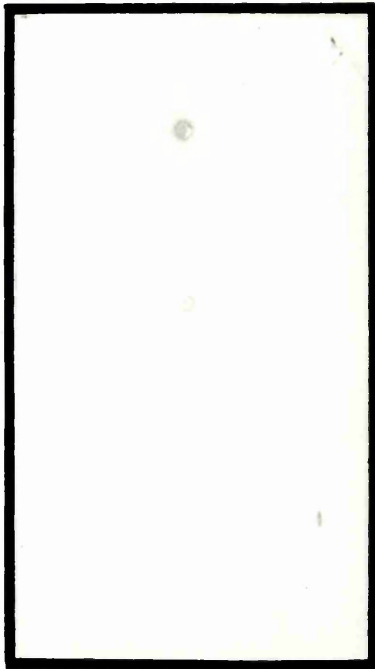
**FIGURE C:** biotinylated secondary antibody (Amersham).

A



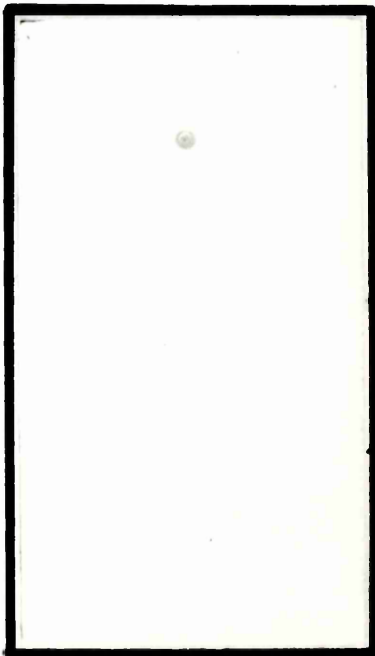
1.0ng  
1.0ng  
0.1ng  
control

B



10ng  
1.0ng  
0.1ng  
control

C



10ng  
1.0ng  
0.1ng  
control

### 3.2 ISOLATION OF CLONES FROM A RAT LIVER cDNA LIBRARY IN $\lambda$ GT11

The preparation of the rat liver cDNA library used in this project is described by Schwarzbauer *et al.*, (1983) and was kindly donated by Dr. R.O. Hynes.

#### 3.2.1 Determination of the titre and number of recombinants within the library

Before the library was screened, the titre of the library i.e. the number of plaque forming units (p.f.u.) / $\mu$ l was determined. Various dilutions of phage from the library were adsorbed to bacteria (*E.coli* strain Y1088), plated out and incubated overnight as described in Section 2.10.4. The number of plaque forming units was counted and the titre was determined to be  $9.7 \times 10^3$  p.f.u./ $\mu$ l (Table 3.2).

The number of recombinant phage within the library was determined also. Phage from the library were adsorbed to *E.coli* strain Y1090, top agar containing isopropyl- $\beta$ -D thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X Gal) was added and the infected bacteria plated out and incubated as described in Section 2.10.4. 56% of the plaques were clear and therefore contained cDNA inserts (Table 3.3)

TABLE 3.2 DETERMINATION OF THE TITRE OF THE cDNA  
LIBRARY IN  $\lambda$ GT11

Phage were taken from the library, diluted in SM buffer, adsorbed to *E.coli* strain Y1088, plated out and incubated as described in Section 2.10.4. The number of plaque forming units (pfu) was then counted and the titre (pfu/ $\mu$ l) determined

Dilutions	p.f.u./plate	p.f.u.x10 <sup>3</sup> /μl	Average p.f.u.x10 <sup>-3</sup> /μl
10 <sup>-4</sup>	130	13	9.7
	109	11	
10 <sup>-5</sup>	7	7	
	8	8	

TABLE 3.3 DETERMINATION OF % RECOMBINANT PHAGE IN  
THE RAT LIVER LIBRARY

Phage were taken from the library, diluted in SM buffer and adsorbed to *E.coli* Y1090. Top agar, containing 2% (w/v) IPTG and 2% (w/v) X-gal was added, the infected bacteria plated out and incubated as described in Section 2.10.4. Blue plaques (non-recombinants) and white plaques (recombinants) were counted and the % recombinants determined.

Dilution	blue plaques	white plaques	total	%recombinants
10 <sup>-3</sup>	84	120	204	59
	102	162	264	61
10 <sup>-4</sup>	37	36	73	48
	45	53	98	54
10 <sup>-5</sup>	4	5	9	55
	6	10	16	62

### 3.2.2 Isolation of cDNA clones from the rat liver cDNA expression library

A detailed description of the procedure for screening the rat liver  $\lambda$ gt11 expression library with antibody probes is given in Section 2.10.2.

Initially, a total of 300,000 recombinants was screened with ribonuclease inhibitor antibodies and eight positive signals were identified on the nitrocellulose filters. Of these eight putative positive signals, five clearly had the characteristic "doughnut" shape i.e. the edges of the positive signal were darker than the area in the middle and it was decided to isolate the plaques corresponding to these positive signals. It was impossible to directly identify the positive plaques on the agar plate due to the density of the plaques, thus the general area corresponding to the positive signal was picked using the broad end of a pasteur pipette. The phages were then purified from the plaques as described in Section 2.10.3. The phages were taken through a second round of screening at a density of 10,000 pfu per 90mm plate. Of the five putative positives isolated from the initial screen, only three produced positive signals in the second round. Again, the plaques corresponding to these positive signals were isolated and screened for a third time at a density of approximately 500 pfu per plate. It was now possible to directly identify the plaques giving rise to a positive signal on the nitrocellulose filter. Thus, a single plaque was picked and the phage rescreened at a density of 100 pfu per plate. Each plaque now gave rise to a positive signal on the filter.



FIGURE 3.11 ISOLATION OF cDNA CLONE,  $\lambda$ RLRI 7 FROM THE RAT LIVER EXPRESSION LIBRARY.

The clone was isolated as described in Section 2.10.2.

FIGURE A: Second round of screening.

10,000 plaque forming units (pfu) were plated out. Arrows show the positions of three distinctly positive signals.

FIGURE B: Third round of screening.

500 pfu were plated out. Approximately 5% of the plaques appear to produce a positive signal.

FIGURE C: Final round of screening.

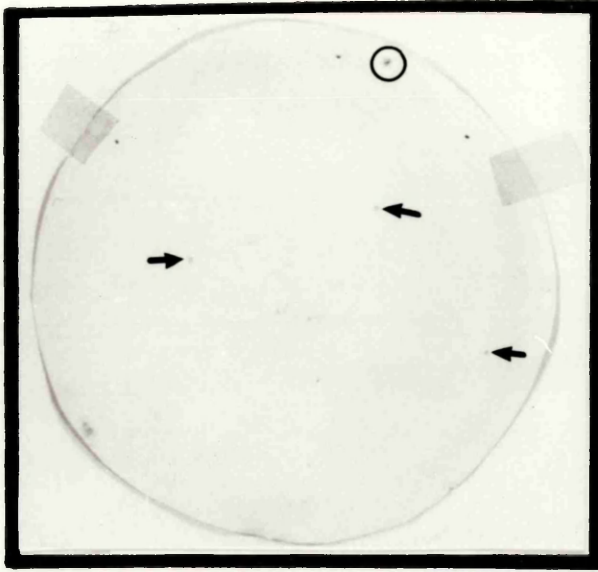
100pfu plated out. Each plaque gave rise to a positive signal.

FIGURE D: Control.

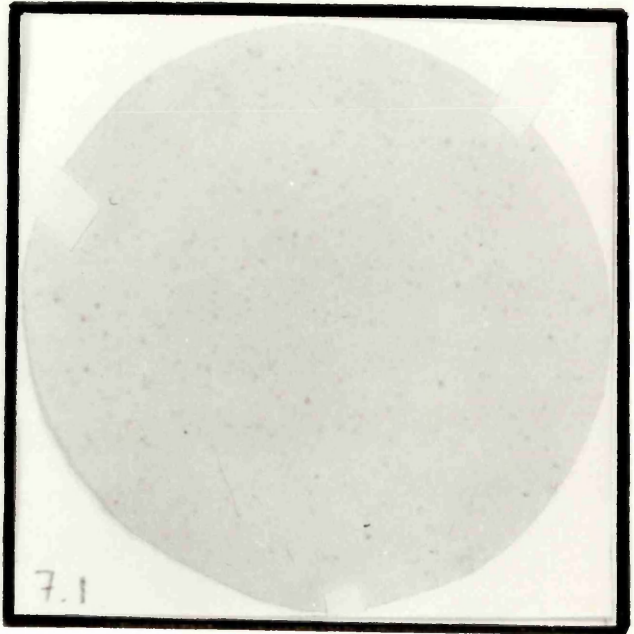
1,000 pfu of non-recombinant  $\lambda$ gt11. No positive signals observed.

The circles in Figures A and D outline the positive control where 5ng of purified ribonuclease inhibitor was spotted directly onto the filter to ensure that the detection system was working.

A



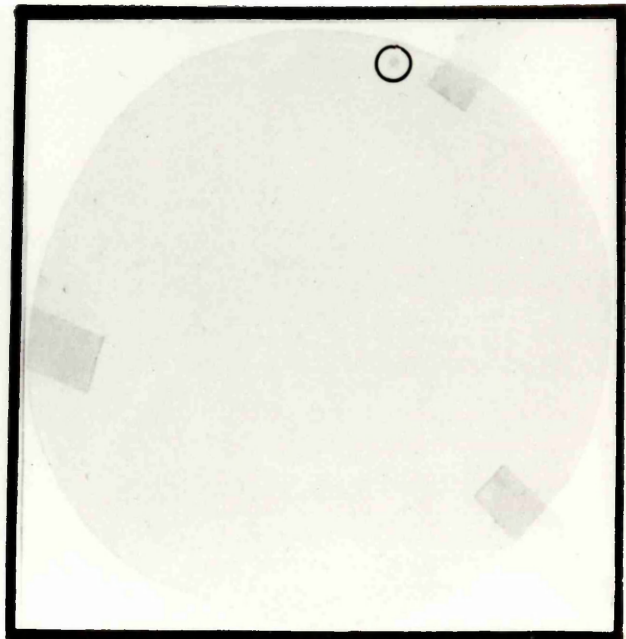
B



C



D



Thus, three clones were isolated and named  $\lambda$ RLRI 3,  $\lambda$ RLRI 7 and  $\lambda$ RLRI 8.

The isolation of  $\lambda$ RLRI 7 through the second, third and final rounds of screening is shown in Figure 3.11. Similar results were obtained in the isolation of  $\lambda$ RLRI 3 and  $\lambda$ RLRI 8.

Several controls were carried out while screening the library to decrease the possibility of obtaining false positives.

During each round of screening, a duplicate nitrocellulose filter was obtained as described in Section 2.10.2. This duplicate filter was screened with IgG purified from pre-immune serum. This control ensured that the positive signals obtained on the filter were produced by the antibody recognising and binding to a specific antigen (i.e. ribonuclease inhibitor) and not merely due to a non-specific association with any expressed proteins. At no time did the pre-immune IgG produce a positive signal on the filters. An example of a filter screened with pre-immune and immune IgG is shown in Figure 3.12.

A second control was the screening of non recombinant  $\lambda$ gt11 with ribonuclease inhibitor antibodies (Figure 3.11 D) to ensure that the antibodies did not bind to antigens produced by  $\lambda$ gt11 itself. Again, a positive signal was never observed with this control.

In the earlier rounds of screening, a third control was also carried out. 1ng of purified inhibitor was spotted onto the edge of the nitrocellulose filters. When the filter was developed, a small dark spot was obtained showing where the antigen had bound to the purified antigen (see Figures 3.11.A and D). Thus, if no other

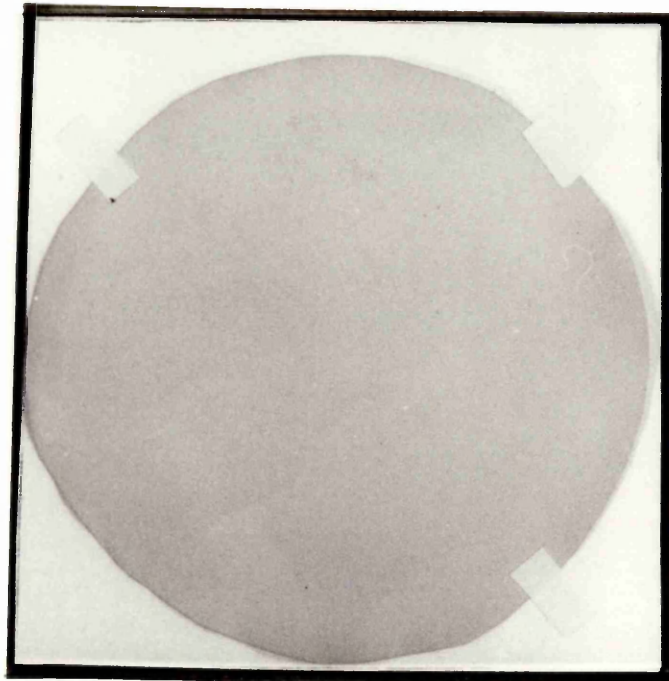
FIGURE 3.12 SCREENING OF ISOLATED CLONE,  $\lambda$ RLRI 7 WITH IMMUNE AND PRE-IMMUNE IgG

Approximately 250 p.f.u. of  $\lambda$ RLRI 7 obtained after the third round of screening were plated out (Section 2.10.1) and the filters probed with either immune serum (Figure A) or pre-immune serum (Figure B). The filters were developed as detailed in Section 2.10.2.

A



B



positive signals were observed on the filter, it was concluded that these plaques did not express a protein which was recognised by the antibody.

### **3.3 CHARACTERISATION OF THE cDNA CLONES ISOLATED FROM THE EXPRESSION LIBRARY**

#### **3.3.1 Preparation of $\lambda$ gt11 recombinant lysogens in Y1089**

$\lambda$ gt11 recombinant lysogens were generated in Y1089 as described in Section 2.11.1. It is considered to be advantageous to produce recombinant lysogens as cloned DNA is more stable when integrated into the bacterial chromosome. Also, preparative amounts of the polypeptide specified by the cloned DNA can be obtained which is of use when characterising the recombinant clones.

Figure 3.13 shows an example of Y1089 lysogenised with one of the putative ribonuclease inhibitor encoding clones,  $\lambda$ RLRI 8. A putative lysogenic colony was streaked out onto an LB plate and incubated at 30°C (a temperature at which the temperature-sensitive repressor is functional) and then replica plated (Section 2.11.1). One plate was incubated at 42°C (Figure 3.13.A) while the other was incubated at 30°C (Figure 3.13.B). The colonies have grown at 30°C but not at 42°C and are thus assumed to be lysogenic.

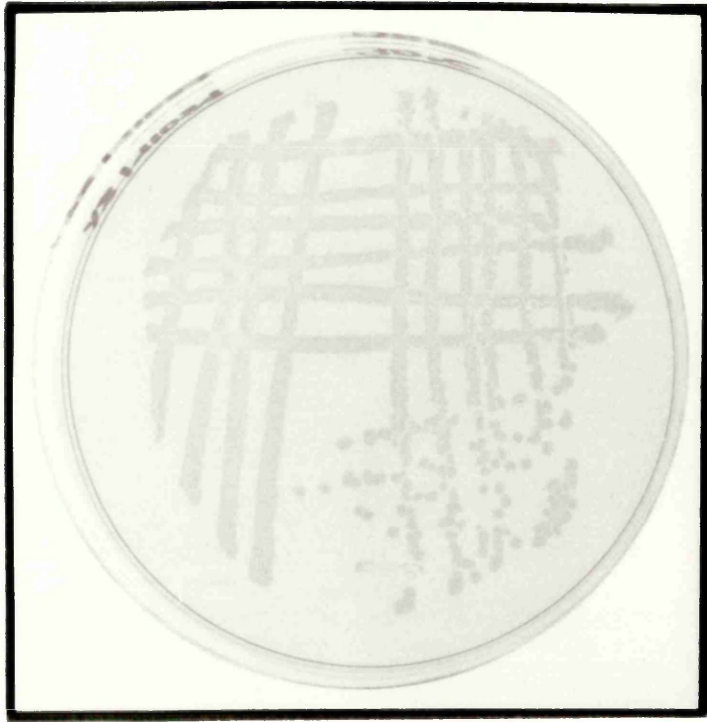
Y1089 was also lysogenised with  $\lambda$ RLRI 3,  $\lambda$ RLRI 7 and non recombinant  $\lambda$ gt11. All were tested for lysogeny as described for  $\lambda$ RLRI 8 with identical results.

**FIGURE 3.13 GENERATION OF A  $\lambda$ GT11 RECOMBINANT  
LYSOGEN IN Y1089**

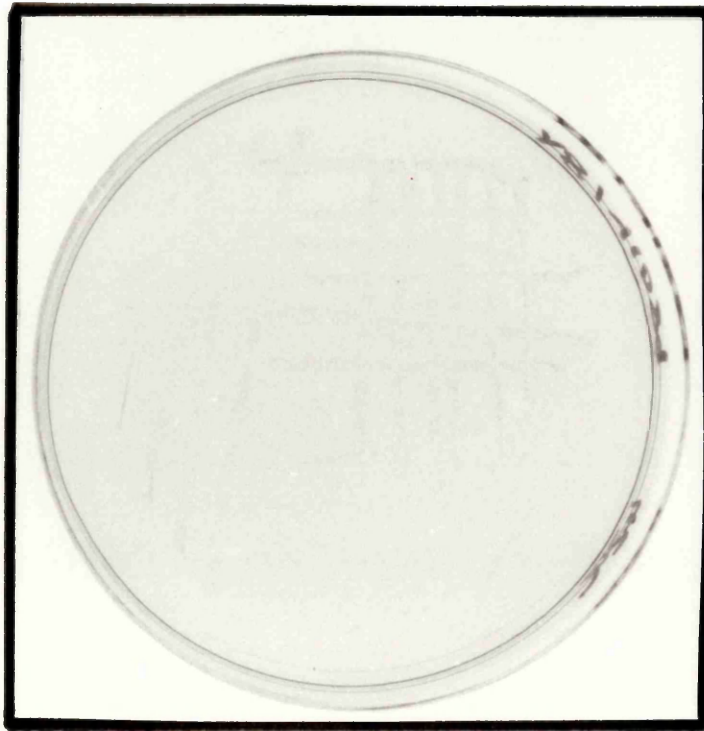
Y1089 was lysogenised with  $\lambda$ RLRI 8 as described in Section 2.11.1. The figure shows replica plates, one of which has been grown at 30°C (Figure A), and the other at 42°C (Figure B). Colonies have grown only on the plate incubated at 30°C.



A



B



### 3.3.2 Characterisation of $\beta$ -galactosidase fusion proteins by SDS PAGE

As the cDNA of recombinant  $\lambda$ gt11 is inserted into the EcoRI site of the  $\beta$ -galactosidase gene, a fusion protein is produced in the presence of IPTG. This fusion protein comprises a large segment of  $\beta$ -galactosidase (114,000 Daltons) fused to protein sequences encoded by the cDNA insert. Each of the recombinant  $\lambda$ gt11 lysogens were treated with IPTG to induce the synthesis of  $\beta$ -galactosidase. Crude cell lysates were then prepared from them and also from a non-recombinant  $\lambda$ gt11 lysogen (Section 2.11.2.). The lysates were analysed for the presence of fusion proteins by SDS PAGE on a 7.5% polyacrylamide gel (Figure 3.14). Fusion proteins were detected in each of the three selected recombinants and those produced from  $\lambda$ RLRI 3 and  $\lambda$ RLRI 8 were dependent on the induction with IPTG. An anomalous result was obtained for the lysogen  $\lambda$ RLRI 7 as fusion protein was synthesised in both the absence and presence of IPTG. (It should be noted that this anomalous result was not reproducible). The  $M_r$  of the fusion proteins were determined as described in Section 2.4.8. The  $M_r$  of the fusion protein produced by the lysogens of  $\lambda$ RLRI 3 and  $\lambda$ RLRI 8 was approximately 127,000 Daltons whereas that from the lysogen of  $\lambda$ RLRI 7 was 130,000 Daltons.

FIGURE 3.14 CHARACTERISATION OF  $\beta$ -GALACTOSIDASE FUSION PROTEINS BY SDS PAGE.

IPTG-induced and non-induced crude cell lysates (prepared as described in Section 2.11.2) were resolved on a 7.5% SDS polyacrylamide gel and stained with Coomassie Blue as outlined in Sections 2.4.3 and 2.4.5.A.

Lane 1: 200 $\mu$ g of IPTG-induced cell lysate from non-recombinant  $\lambda$ gt11 lysogen.

Lane 2: 200 $\mu$ g of uninduced crude cell lysate from the lysogen of  $\lambda$ RLRI3.

Lane 3: 200 $\mu$ g of IPTG-induced crude cell lysate from the lysogen of  $\lambda$ RLRI 3.

Lane 4: 200 $\mu$ g of uninduced crude cell lysate from the lysogen of  $\lambda$ RLRI7.

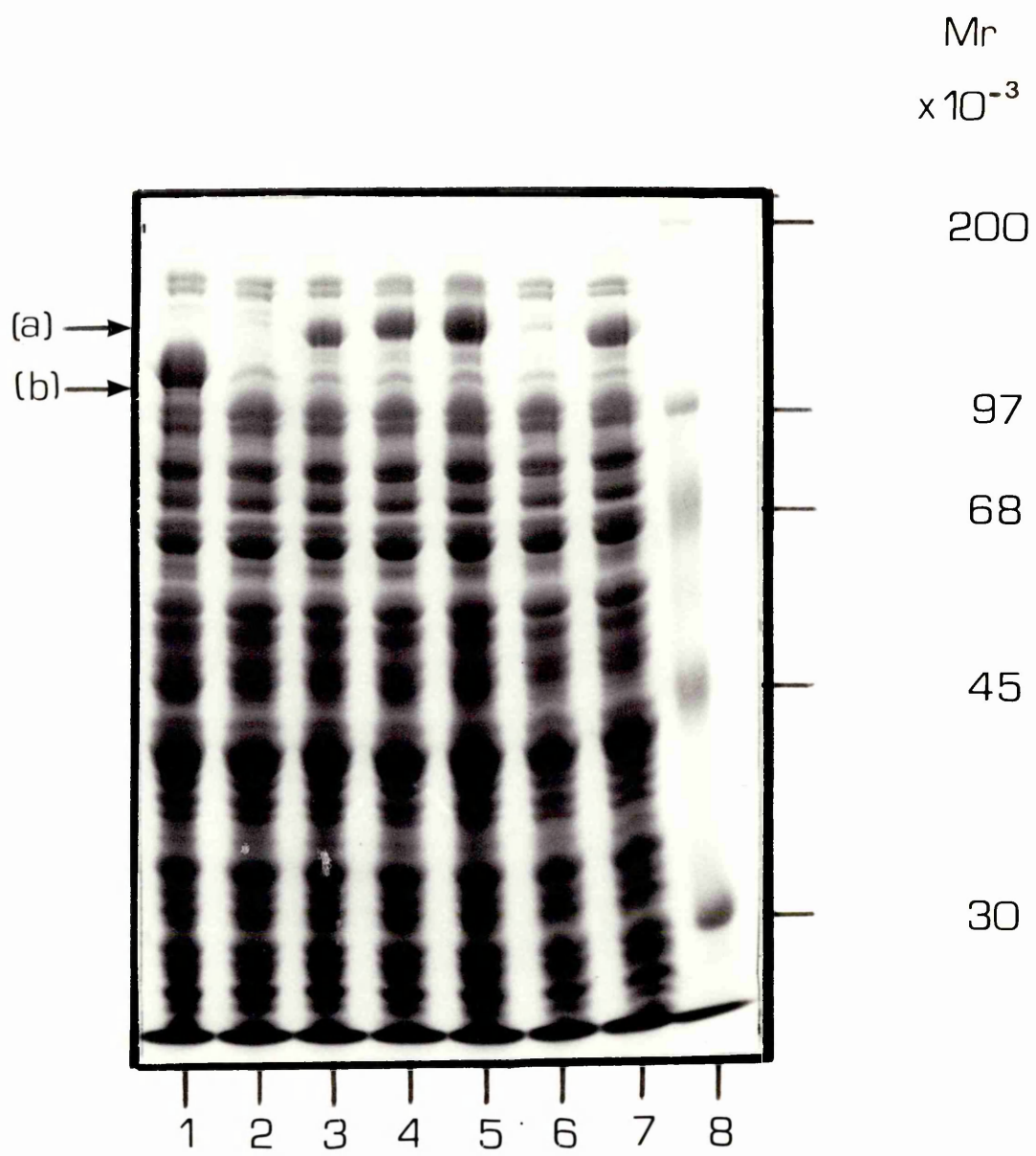
Lane 5: 200 $\mu$ g of IPTG-induced crude cell lysate from the lysogen of  $\lambda$ RLRI 7.

Lane 6: 200 $\mu$ g uninduced crude cell lysate from the lysogen of  $\lambda$ RLRI8.

Lane 7: 200 $\mu$ g of IPTG-induced crude cell lysate from the lysogen of  $\lambda$ RLRI 8.

Lane 8: Molecular weight markers as described in Figure 3.2.

The arrow at (a) shows the position of the induced fusion proteins and at (b) indicates the position of non-recombinant  $\beta$ -galactosidase.



### 3.3.3 Characterisation of $\beta$ -galactosidase proteins by western blotting

The fusion proteins detected as described above were further analysed by western blotting. Crude cell lysates were subjected to electrophoresis on a 7.5% SDS polyacrylamide gel, blotted onto nitrocellulose and challenged with ribonuclease inhibitor antiserum as described in Section 2.7.3. When the blot was developed with the horseradish peroxidase conjugated second antibody system, no immune positive bands were observed. However, when developed with the biotin streptavidin system (Amersham), an immune positive band was observed for  $\lambda$ RLRI 7 and  $\lambda$ RLRI 8 in the IPTG-induced cell lysates. No immune positive band was observed for  $\lambda$ RLRI 3. It was thus decided to discontinue characterising  $\lambda$ RLRI 3. Figure 3.15.A represents a western blot of non-induced and IPTG-induced crude cell lysates of  $\lambda$ RLRI 7 (lanes 1 and 2) and  $\lambda$ RLRI 8 (lanes 3 and 4) and induced crude cell lysate of non-recombinant  $\lambda$ gt11 (lane 5) when challenged with immune serum. Figure 3.15.B shows the same western blot when challenged with pre-immune serum. These blots demonstrate the presence of ribonuclease inhibitor antigenic determinants within the fusion protein. The antibodies are not recognising  $\beta$ -galactosidase antigenic determinants as an immune band is not observed in the lane containing induced non-recombinant  $\beta$ -galactosidase (lane 5). The additional bands which are observed in Figure 3.15.A are not the result of an immune response as they are also present in the western blot challenged with pre-immune serum (Figure 3.15.B)

FIGURE 3.15 WESTERN BLOT ANALYSIS OF  $\beta$ -GALACTOSIDASE  
FUSION PROTEINS

Crude cell lysates (prepared as described in Section 2.11.2) were electrophoresed on a 7.5 % polyacrylamide gel, blotted onto nitrocellulose and challenged with either immune serum (Figure A) or pre-immune serum (Figure B) as outlined in Sections 2.4.3 and 2.7.3. The western blot was developed using the biotin-streptavidin detection system.

On both Figures A and B, each lane contains 200 $\mu$ g of protein from the appropriate crude cell lysate as follows:

lane 1: uninduced lysate from the  $\lambda$ RLRI 7/Y1089 lysogen;

lane 2: IPTG-induced lysate from the  $\lambda$ RLRI 7/Y1089 lysogen;

lane 3: uninduced lysate from the  $\lambda$ RLRI 8/Y1089 lysogen;

lane 4: IPTG-induced lysate from the  $\lambda$ RLRI 8/Y1089 lysogen;

lane 5: IPTG-induced lysate from the non-recombinant  $\lambda$ gt11/Y1089 lysogen.

A

B



#### 3.3.4 Determination of the % concentration of fusion protein in a crude cell lysate.

To estimate the amount of fusion protein present in a crude cell lysate, samples of IPTG-induced and non-induced crude cell lysate (prepared as described in Section 2.11.2) were subjected to SDS polyacrylamide gel electrophoresis (Section 2.4.3) on a 7.5% (w/v) polyacrylamide gel, then stained with Coomassie Blue (Section 2.4.5.A). After destaining, both tracks were scanned on a LKB 'multiscan' and a characteristic trace obtained. Each peak on the trace corresponds to a band of protein on the gel. A diagrammatic representation of the absorbance trace for the crude cell lysate containing the fusion protein from  $\lambda$ RLRI 8 is shown in Figure 3.15. The additional peak in trace B corresponds to the fusion protein. The area under each peak (curve) was calculated by computer and expressed as a percentage of the total area (Table 3.4). This analysis revealed that the area under the curve representing the fusion protein was approximately 16% of the total area. Hence the fusion protein represents approximately 16% of the Coomassie Blue stained protein present in the crude cell lysate.



FIGURE 3.16 SCAN OF UNINDUCED AND IPTG-INDUCED  
CRUDE CELL LYSATES ON SDS POLYACRYLAMIDE GELS

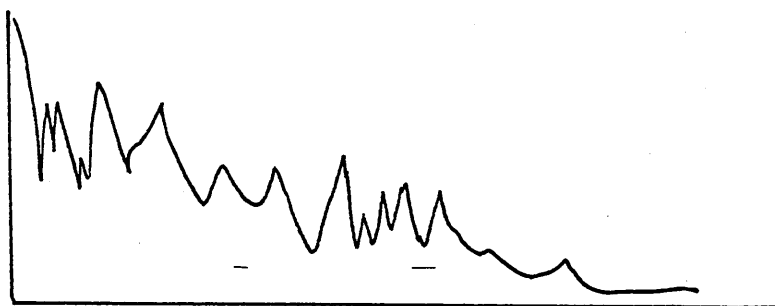
Crude cell lysates of the lysogen  $\lambda$ RLRI 8/Y1089 (described in Section 2.11.2) were subjected to electrophoresis on a 7.5% polyacrylamide gel (Section 2.4.3), stained with Coomassie Blue and destained as described in Section 2.4.5.A. Individual tracks on the gel were analysed on an LKB 'multiscan' to determine the percentage of fusion protein in the total protein preparation.

Figure A is representative of the trace obtained when the uninduced lysate was scanned.

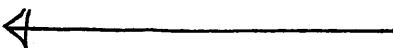
Figure B is representative of the trace obtained when the IPTG-induced cell lysate was scanned.

The peak corresponding to the fusion protein is shown by the arrow (15)  
The percentage of total protein represented by each peak is outlined in the following table (Table 3.4)

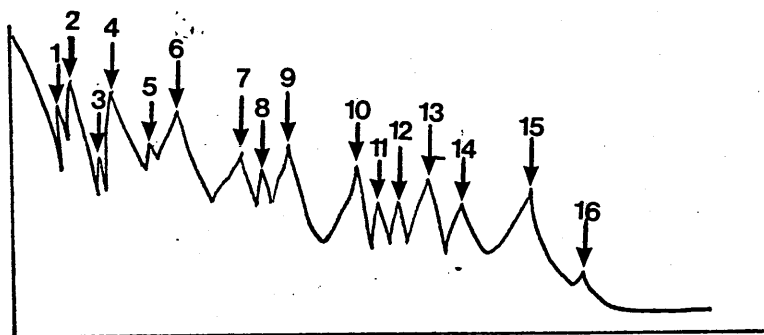
A



⊕ Migration of proteins on SDS PAGE ⊖



B



⊕ Migration of proteins on SDS PAGE ⊖

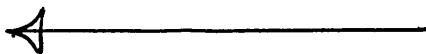


TABLE 3.4 DETERMINATION OF THE AMOUNT OF FUSION  
PROTEIN IN AN IPTG-INDUCED CRUDE CELL LYSATE

The peaks of protein shown in Figure 3.14.B are presented here as a percentage of the total protein in the crude cell lysate.

Peak 15 represents the fusion protein and is approximately 16% of the total protein.

Peak number	% Area
1	1.8
2	5.3
3	0.7
4	6.3
5	1.0
6	17.6
7	5.6
8	1.7
9	5.5
10	9.3
11	3.2
12	5.6
13	8.5
14	10.5
15	16.2
16	1.2

### 3.3.5 Comparison of peptide maps of rat liver ribonuclease inhibitor and fusion protein

After isolation of a cDNA clone, the subsequent analysis must ensure that it encodes the protein of interest. In general, the nucleotide sequence of the cDNA is obtained and, from this, the predicted amino acid sequence of the protein encoded by the clone is revealed. Then, if the amino acid sequence of the protein of interest is available, the predicted amino acid sequence and the authentic sequence are compared. However, at this stage in the project i.e. after the clones were isolated from the library, neither amino acid sequence nor nucleotide sequence of any ribonuclease inhibitor were available. A comparison of the sequences was thus impossible. Instead, a novel approach was used to determine if the clones  $\lambda$ RLRI 7 and  $\lambda$ RLRI 8 encoded the ribonuclease inhibitor using a combination of peptide mapping and western blotting.

When a protein is digested with a protease and the products separated on SDS PAGE, a characteristic pattern of bands is obtained. This banding pattern is known as a peptide map. If two proteins are similar and both are digested with the same protease, similar peptide maps are obtained. Thus, it was decided to compare the peptide maps obtained after digestion of purified ribonuclease inhibitor and fusion protein. It was hoped that partial identity in the peptide maps would indicate that the cDNA encoded the inhibitor. However, digestion of the  $\beta$ -galactosidase fusion protein with any protease leads to the generation of many peptides: some derived from the  $\beta$ -galactosidase portion of the gene; others from the

FIGURE 3.17 IMMUNOBLOT ANALYSIS OF RIBONUCLEASE  
INHIBITOR DIGESTED WITH CHYMOTRYPSIN

The protein was digested with differing amounts of chymotrypsin at 37°C for two hours. The digested products were subjected to electrophoresis on a 15% polyacrylamide gel (Section 2.4.3), then transferred onto nitrocellulose and challenged with immune serum (Section 2.7.3). The blot was developed with the HRP conjugated secondary antibody system (SAPU) as described in Section 2.7.3.

Each lane contains inhibitor digested with chymotrypsin at a ratio of protein:protease as outlined below:

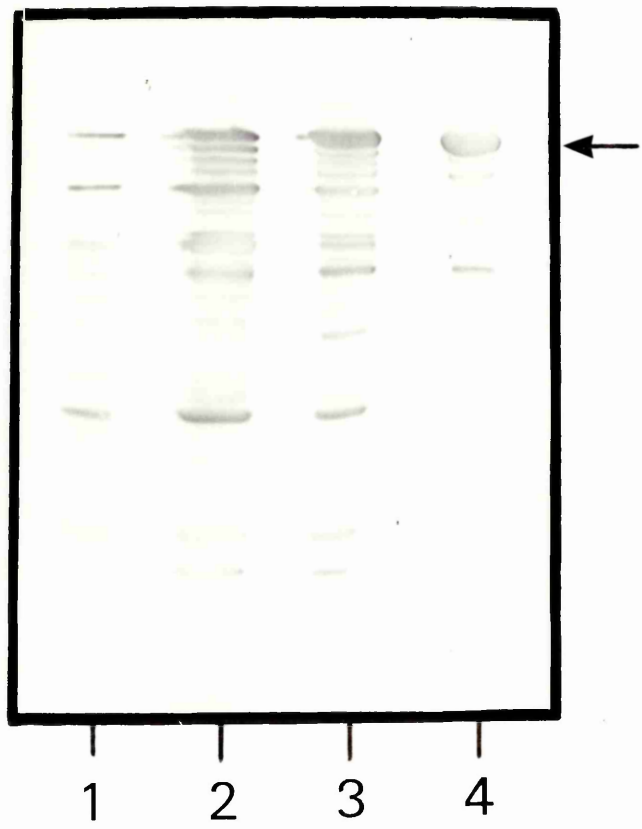
lane 1: 10:1;

lane 2: 100:1;

lane 3: 1000:1;

lane 4: undigested ribonuclease inhibitor.

The arrow shows the position of undigested inhibitor.



protein encoded by the cDNA. Thus, to distinguish cDNA encoded peptides from  $\beta$ -galactosidase peptides, the digested products were blotted onto nitrocellulose filters and challenged with ribonuclease inhibitor antiserum. Peptides obtained from digestion of purified inhibitor were similarly analysed and compared with antigenic fragments of the fusion protein.

Initially a solution of purified ribonuclease inhibitor was digested with varying amounts of chymotrypsin. The products of digestion were subjected to electrophoresis on a 15% SDS polyacrylamide gel (Section 2.4.3), transferred onto nitrocellulose and challenged with immune serum (Section 2.7.3). The result of the immunoblot after this procedure is shown in Figure 3.17. The protein has been digested with chymotrypsin and a clear, distinctive immunogenic peptide map obtained (the additional bands seen in the lane containing undigested inhibitor may be due to either degradation of the preparation during storage or contamination with chymotrypsin while loading the gel).

Various attempts were made to isolate the fusion protein from a crude cell lysate so that protease digestion could be performed on purified protein. It has been reported that a large number of genes expressed as fusion proteins in the cytoplasm of *E.coli* are insoluble (Harris, 1983) and accumulate in a discrete form known as "inclusion bodies" (Williams *et al.*, 1982). Inclusion bodies are dense and sediment readily with low speed centrifugation e.g. 5,000-12,000x  $g_{av}$ . Under these conditions, the inclusion bodies sediment more rapidly than the bulk of cell debris, thus allowing the purification of the fusion protein. Figure 3.18 shows that the fusion proteins from the lysogens  $\lambda 7/Y1089$  and  $\lambda 8/Y1089$  were



FIGURE 3.18 SDS PAGE ANALYSIS OF THE SOLUBILITY OF FUSION PROTEIN FROM THE LYSOGEN OF  $\lambda$ RLRI 8/Y1089

Harvested IPTG-induced bacterial cells were resuspended in 50mM Tris.HCl pH7.5 containing different salt concentrations as follows: 0.15M NaCl (lanes 1 and 2); 0.5M NaCl (lanes 3 and 4) or 2M NaCl (lanes 5 and 6). The cells were lysed by repeated heating of the samples to 37°C, then plunging them into a dry ice/methanol bath. The lysates were subjected to low speed centrifugation and the resultant pellets and supernatant fractions analysed by SDS PAGE (Section 2.4.3), then stained with Coomassie Blue and destained as described in Section 2.4.5.A.

lane 1: 100 $\mu$ g of supernatant fraction from a non-induced lysate of  $\lambda$ RLRI 8/Y1089 lysogen.

lane 2 : 100 $\mu$ g of supernatant fraction from IPTG-induced lysate of  $\lambda$ RLRI 8/Y1089 lysogen.

\*lane 3: resuspended pellet from a  $\lambda$ RLRI 8/Y1089 IPTG-induced culture lysed in the presence of 0.5M NaCl.

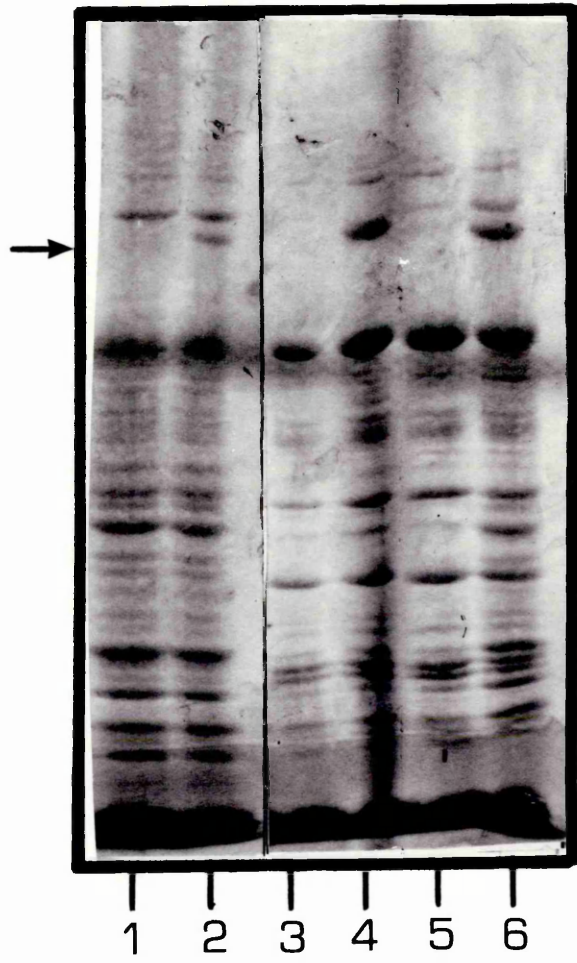
lane 4: 100 $\mu$ g of supernatant fraction from a  $\lambda$ RLRI 8/Y1089 IPTG-induced culture lysed in the presence of 0.5M NaCl.

\*lane 5: resuspended pellet from a  $\lambda$ RLRI 8/Y1089 IPTG-induced culture lysed in the presence of 2M NaCl.

lane 6: 100 $\mu$ g of supernatant fraction from a  $\lambda$ RLRI 8/Y1089 IPTG-induced culture lysed in the presence of 2M NaCl.

\* The pellets were resuspended in 500 $\mu$ l Laemmli sample buffer and 50 $\mu$ l loaded onto the gel.

Lanes 1 and 2 are controls which show the position of the induced fusion protein (shown by arrow)



soluble as they were present in the supernatant fraction of the cell lysate after centrifugation at  $10,000 \times g_{av}$ . It was thought that by altering either the ionic strength or the pH of the buffers in which the cells were lysed might change the solubility of the fusion protein, thus allowing rapid purification. Figure 3.18 shows the result obtained when the cells were lysed in 50mM Tris.HCl pH7.5, 150mM NaCl (lane 2), in 50mM Tris.HCl pH7.5 buffer containing 0.5M NaCl (lanes 3 and 4) or 2M NaCl (lanes 5 and 6). Lane 1 contained the supernatant fraction from uninduced lysate to allow identification of the position of the fusion protein. After centrifugation of the lysate, the fusion protein was always left in the supernatant fraction. Similar results were observed when lysis was performed in 50mM sodium acetate pH5 or 50mM Tris.HCl pH9 (data not shown). Thus this potentially rapid method of isolating the fusion proteins was unsuccessful. Other methods which were attempted centred on attempting to recover the fusion protein from polyacrylamide gels after their fractionation from other bacterial proteins. Numerous published methods of diffusion or electrophoretic recovery were attempted. All were unsuccessful, presumably because of the very high relative mass of the protein.

As it was proving difficult to isolate the fusion protein, it was decided to use the method of Cleveland *et al.*, (1977) to digest the protein within polyacrylamide and then to perform a western blot on the digested products as described above. Cleveland mapping involves enzymatic digestion of purified proteins in an SDS polyacrylamide gel. Again, the fusion protein was fractionated from the other proteins in a crude cell lysate by electrophoresis and the

FIGURE 3.19 IMMUNOBLOT ANALYSIS OF RIBONUCLEASE  
INHIBITOR AND FUSION PROTEIN DIGESTED WITH  
CHYMOTRYPSIN BY THE CLEVELAND METHOD.

Gel slices of ribonuclease inhibitor, fusion protein and  $\beta$ -galactosidase were obtained and digested with chymotrypsin as described (Section 2.4.7). After electrophoresis, the digested products were analysed by western blotting (Section 2.7.3). The blot was developed using the HRP conjugated second antibody system from SAPU.

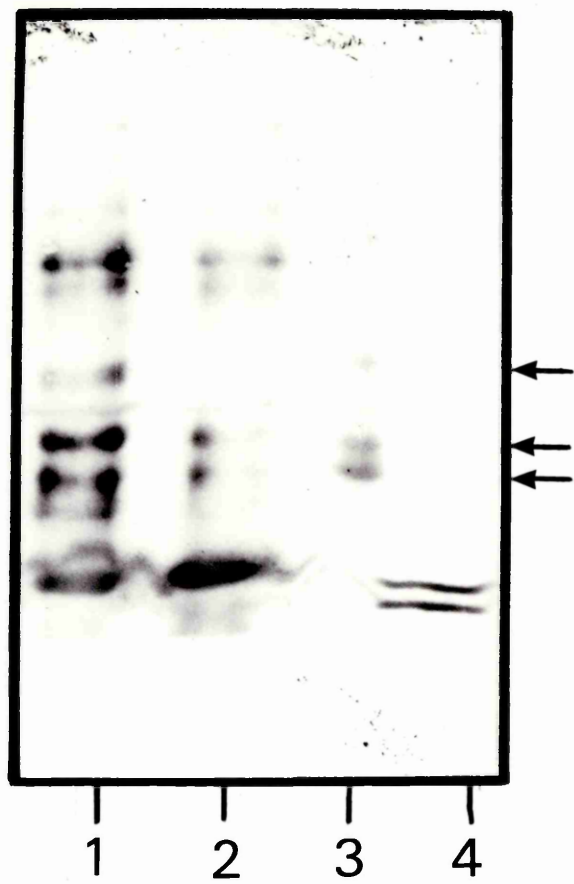
lane 1: 10 $\mu$ g inhibitor digested with 1 $\mu$ g chymotrypsin.

lane 2: 10 $\mu$ g inhibitor digested with 2 $\mu$ g chymotrypsin.

lane 3: 100 $\mu$ g fusion protein digested with 10 $\mu$ g chymotrypsin.

lane 4: 100 $\mu$ g  $\beta$ -galactosidase digested with 10 $\mu$ g chymotrypsin.

The arrows show possible similar peptide bands occurring after digestion of inhibitor and fusion protein with chymotrypsin.



band corresponding to the fusion protein was excised. The gel band was then cut into small pieces and placed in the wells of an SDS polyacrylamide gel for digestion with chymotrypsin as described in Section 2.4.7. A band of ribonuclease inhibitor was excised from a preparative gel and treated in the same manner. After variable periods of digestion, the digested products were separated by electrophoresis on the polyacrylamide gel, the products transferred to nitrocellulose and challenged with ribonuclease inhibitor antiserum. A typical result is shown in Figure 3.19. This figure shows the results obtained when inhibitor and fusion protein were digested with different amounts of chymotrypsin and it is evident that digestion of both proteins has occurred. However, this method did not produce as clear a peptide map as that obtained in Figure 3.17 and all the data obtained, both with chymotrypsin and with V8 protease, was difficult to interpret. It is possible that the peptide bands marked by arrows in Figure 3.19 correspond to similar peptides but the evidence is not clear cut.

### **3.4 AMINO ACID SEQUENCE ANALYSIS OF RAT LIVER RIBONUCLEASE INHIBITOR**

As the comparison of immunogenic peptide maps of fusion protein and ribonuclease inhibitor (Section 3.3.5) had failed to confirm the identity of the clones isolated from the library, it was decided to obtain as much amino acid and nucleotide sequence data as possible and then compare the predicted amino acid sequence obtained from the nucleotide sequence with the amino acid sequence of the purified protein.

The cDNA used to construct this library was prepared using oligo (dT) which creates a bias towards cloning the 3' end of the mRNA. As the clones isolated from the cDNA library were not full length i.e. they were not of sufficient size to encode the entire protein, it was assumed that they probably encoded the 3' non coding region and the COOH-terminal end of the protein. Thus, rather than attempting to sequence the protein from the N-terminus, it was decided to generate peptides by digestion of the protein with various proteases and to obtain sequence data on the peptides. It was hoped that this strategy would generate sequence close to the COOH-terminus and overlapping the amino acid sequence predicted from the nucleotide sequence of the cDNA.

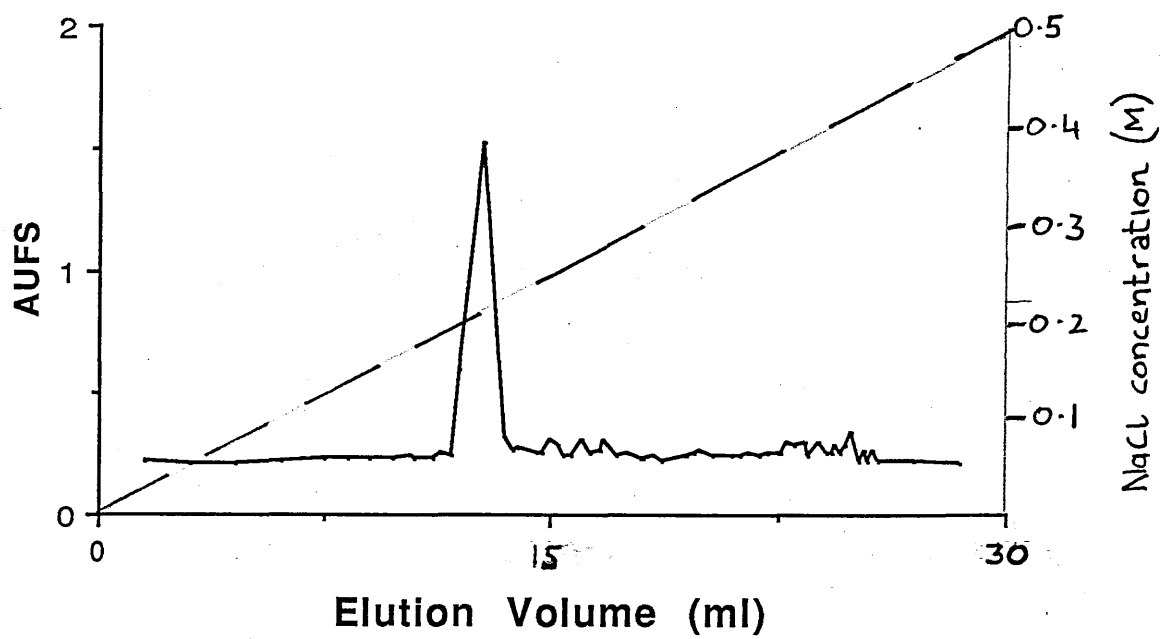
#### **3.4.1 Further purification of ribonuclease inhibitor**

As described in Section 3.1.1, ribonuclease inhibitor was purified from rat liver by the method of Burton *et al.*, (1980) (see

**FIGURE 3.20 FPLC PURIFICATION OF AFFINITY PURIFIED  
RIBONUCLEASE INHIBITOR**

Ribonuclease inhibitor, obtained after affinity chromatography on the RNase A-Sepharose column, was subjected to ion-exchange chromatography on the Mono Q column of an FPLC (Pharmacia) as described in Section 2.5.3. Bound protein was eluted with a NaCl gradient as shown. Inhibitor eluted at approximately 0.3M NaCl. The elution of the protein was measured by absorbance at 280nm with a full-scale deflection from 0 to 2.0.

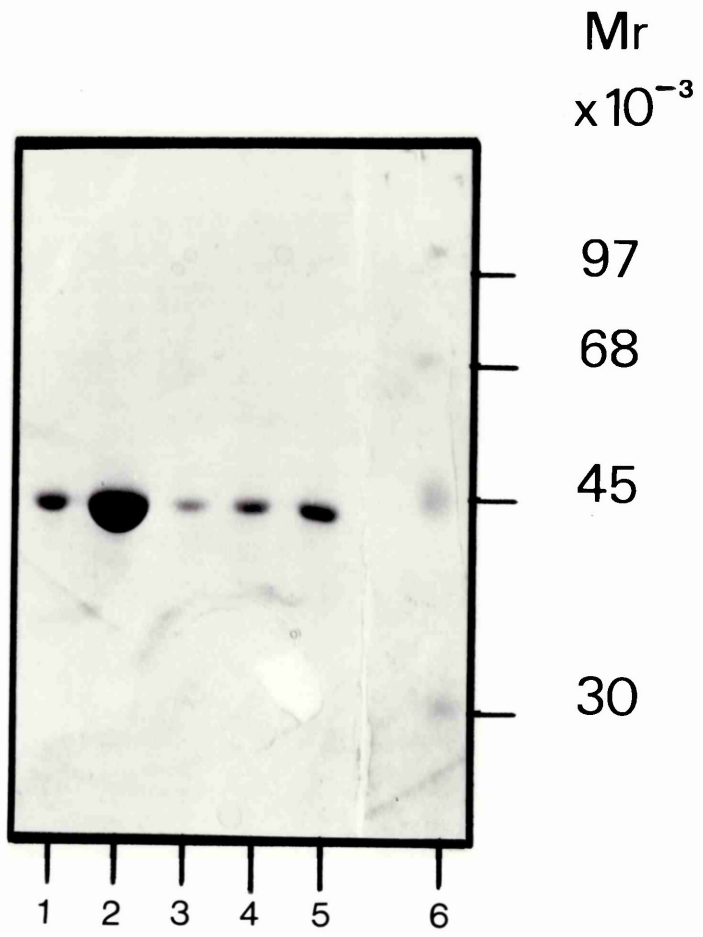




**FIGURE 3.21 ANALYSIS OF RIBONUCLEASE INHIBITOR BY  
SDS PAGE AFTER PURIFICATION ON FPLC**

An aliquot (1/200) of the fractions containing the peak of protein eluted from the Mono Q column (see Figure 3.20) were subjected to electrophoresis on a 10% (w/v) polyacrylamide gel (Section 2.4.3) and protein was visualised by the silver staining method described in Section 2.4.5.B.

Lanes 1 to 5 inclusive are the fractions which eluted from the column with a significant absorbance reading at 280nm. Lane 6 contains molecular weight markers as described in Figure 3.2.



Section 2.5.2) When the purified protein was subjected to SDS PAGE and stained with Coomassie Blue (Figure 3.1), small amounts of contaminating proteins were observed. As the protein was required to obtain amino acid sequence, it was essential to remove these contaminants. The protein which eluted from the RNase A-Sepharose affinity column was subjected to ion-exchange chromatography using the Mono-Q column of the Pharmacia FPLC system as described in Section 2.5.3. Ribonuclease inhibitor was eluted in a sharp peak at approximately 30% (0.3M) NaCl (Figure 3.20) and smaller peaks of protein were eluted at higher salt concentrations. The purity of the protein isolated by this procedure was determined by SDS PAGE and the gel stained with silver as described in Section 2.4.5.A. Silver staining is a more sensitive protein detection system than Coomassie Blue staining and trace amounts of proteins can be visualised. The result of the silver-stained gel is shown in Figure 3.21. The protein preparation appeared to be free from all contaminating proteins and was, therefore, considered to be suitable for preparing peptides for amino acid sequence analysis.

### 3.4.2 Isolation of tryptic and chymotryptic peptides of ribonuclease inhibitor by HPLC

The ribonuclease inhibitor isolated by FPLC as described in the previous section (Section 3.4.2) was digested with either trypsin or chymotrypsin as outlined in section 2.6.2. An aliquot of the digested protein was subjected to chromatography using the reverse phase column (C18) of the HPLC system (Beckman) to separate the resultant peptides. In general, peptides of a hydrophilic nature elute from a reverse-phase column before the hydrophobic peptides. The elution profiles obtained for the protein digested with trypsin (Figure 3.22) and with chymotrypsin (Figure 3.23) are shown. It was decided to collect the peaks (i.e. peptides) which appeared to be well-separated and sharp. These are pointed out in Figures 3.22 and 23 by arrows. The remaining digested protein was applied to the C18 column and the peaks collected by hand using the original traces as a guide to their elution time. In total, 13 peptides were collected.

The purity of the peptides was determined by applying an aliquot of each peptide to the C18 column with a buffering system with a different pH (Section 2.6.3). Of the 13 original peptides, only five were represented by a single peak on the second system. These were taken to Aberdeen University to be sequenced on the SERC gas phase sequencer machine.

FIGURE 3.22 PURIFICATION BY HPLC OF PEPTIDES  
PRODUCED BY DIGESTION OF RIBONUCLEASE INHIBITOR  
WITH TRYPSIN.

An aliquot (1/10) of the digested protein (Section 2.6.2) was applied to the C18 column of a Beckman HPLC. Peptides were eluted with a 0-70% gradient of acetonitrile and were monitored by absorption at 220nm. The absorbance units full scale (AUFS) were from 0 to 0.2. Peptides which were collected are indicated by arrows and were denoted T1 to T7.

The peak at A was caused by injection of the sample into the column and the peak at B was assumed to be protein which did not bind to the column.

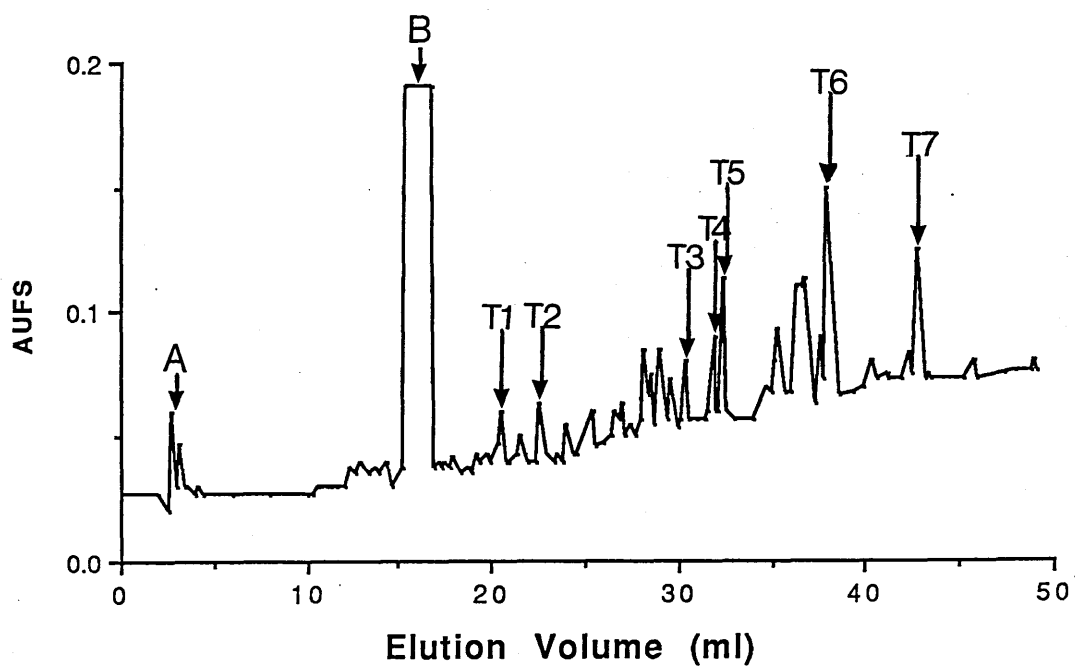
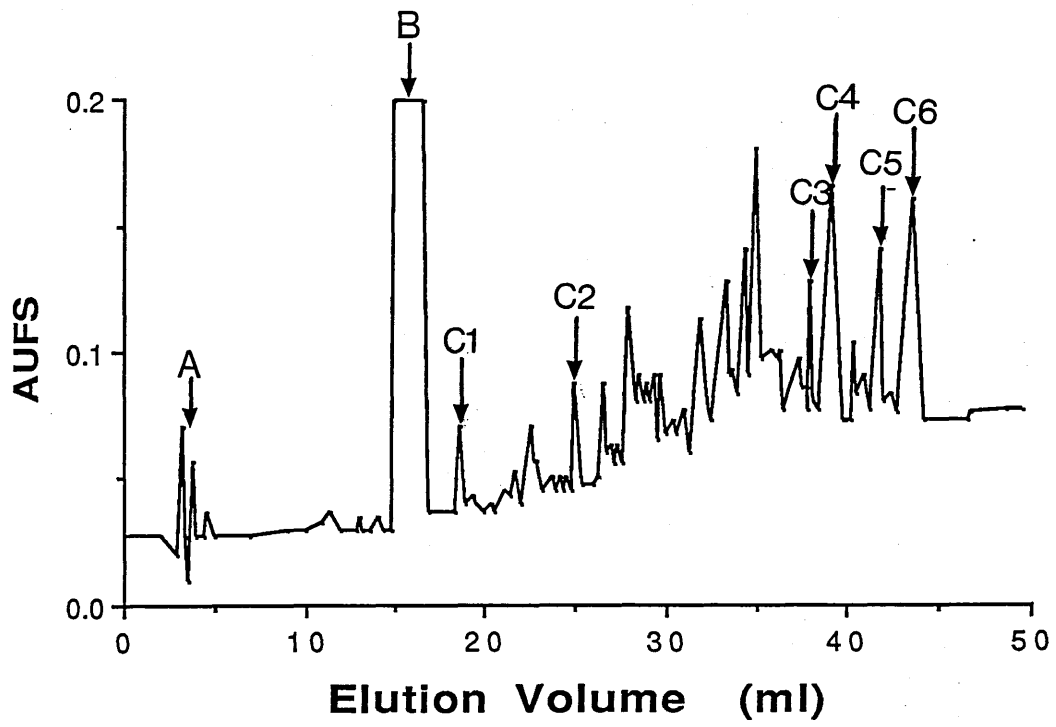


FIGURE 23 PURIFICATION BY HPLC OF PEPTIDES PRODUCED  
BY DIGESTION OF RIBONUCLEASE INHIBITOR WITH  
CHYMOTRYPSIN.

1/10 of the protein digested with chymotrypsin (Section 2.6.2) was subjected to chromatography on a C18 reverse phase column of a Beckman HPLC. The peptides were eluted with a 0-70% acetonitrile gradient and were monitored by absorption at 220nm with AUFS from 0 to 0.2. The arrows show the peaks (peptides) which were collected. These peptides were denoted C1 to C6 inclusive.

(A) represents the injection spike and (B) was assumed to be the protein which did not bind to the column.





### 3.4.3 Gas phase sequencing of the peptides

The five peptides isolated by HPLC purification were sequenced on an automatic gas phase sequencer as described in Section 2.6.3. The first peptide to be sequenced (T6) provided a total of 11 residues. The yields of residues recovered from each cycle of the sequence is shown in Table 3.5. Sequencing was continued until no other PTH-amino acid derivatives could be identified above background level. As can be seen from this table, the sequence of the peptide included two successive glutamine residues (residues 9 and 10). Unfortunately, this apparently quenched the sequencing reaction and hence no further sequence was obtained from this peptide (B. Dunbar, personal communication).

Disappointingly, the other peptides yielded no sequence data at all. It was suggested that there were insufficient amounts of these peptides to allow sequencing, although HPLC data indicated that the recovery of at least some of them should have been more than adequate. The amino acid compositions of the peptides were determined (shown in Table 3.6) and this also revealed that there should have been sufficient amounts of the peptides to allow them to be sequenced.

**TABLE 3.5 AMINO ACID SEQUENCE ANALYSIS OF PEPTIDE T6**

Peptide T6, prepared and isolated as described in Section 2.6.3, was applied to a Beckman automatic amino acid sequence analyser (operated by B.Dunbar, Department of Biochemistry, University of Aberdeen).

SEQUENCE NUMBER	RESIDUE	pMOLES RECOVERED
1	LEU	107.5
2	THR	27.0
3	GLU	22.5
4	LEU	32.0
5	GLY	9.0
6	PRO	20.0
7	LEU	17.5
8	ILE	22.5
9	GLN	20.3
10	GLN	24.0
11	TYR	6.3

TABLE 3.6 AMINO ACID COMPOSITION OF TRYPTIC AND CHYMOTRPTIC PEPTIDES

The peptides, C1, C2, T1, T5 and T6 were hydrolysed in 6M HCl overnight, then applied to a Beckman amino acid analyser (operated by I.McDonald, Department of Biochemistry, University of Aberdeen). Glutamine and glutamate residues are both included in GLU and asparagine and aspartate are both included in ASP.

Cys AC, SCM Cys and PE Cys are derivatives of cysteine residues.

Tryptophan was not detected.

AMINO ACID	pMOLES RECOVERED FROM PEPTIDE				
	C1	C2	T1	T5	T6
CYS AC	0	0	0	0	0
ASP	84	732	192	1236	816
GLU	180	588	576	1632	1692
SCM CYS	0	0	0	0	0
SER	168	492	324	1536	1140
GLY	432	852	672	1248	1668
HIS	0	276	0	132	84
ARG	156	264	516	588	456
THR	120	240	240	1020	1056
ALA	228	828	492	972	1056
PRO	48	72	132	516	780
TYR	48	48	36	228	288
VAL	156	480	240	816	744
MET	0	0	0	60	60
PE CYS	0	0	0	0	0
ILE	156	396	120	612	696
LEU	240	600	1572	1536	3264
PHE	96	336	72	276	192
LYS	120	228	132	480	288
Total pmoles	2232	6432	5316	12888	14280

### **3.5 DNA SEQUENCE ANALYSIS OF cDNA CLONES**

#### **3.5.1 Subcloning of the rat liver cDNA clones**

The cDNA inserts from the clones  $\lambda$ RLRI 7 and  $\lambda$ RLRI 8 were subcloned into the multifunctional vector, pTZ as described in Section 2.13. The lambda DNA, purified as described in Section 2.12.1, was digested with EcoR1 and the digested products analysed by agarose gel electrophoresis. This revealed that both clones contained an insert of less than 500bp (data not shown). The EcoR1-digested lambda DNA was ligated to EcoR1-digested vector with a molar ratio of insert:vector of 3:1 i.e. 2.2 $\mu$ g digested l DNA was ligated with 300ng vector DNA. The ligated DNA was then transformed into competent DS941 bacteria (Section 2.13.5). Positive (white) colonies on LB agar plates containing ampicillin, IPTG and Xgal were taken through two successive rounds of purification to ensure that they were not false positives. Plasmid DNA was purified from the positive colonies by the mini method as described in Materials and Methods (Section 2.12.3), digested with EcoR1 and subjected to agarose gel electrophoresis to determine the size of the cDNA insert. The results of the EcoR1 digestion are shown in Figure 3.24 and the position of the cDNA inserts are marked by arrows. The size of the inserts was determined using the 123bp ladder as a standard (see Section 2.17.2) and found to be approximately 371bp for the insert from pRLRI 7 and 316bp for the insert from pRLRI 8. The ribonuclease inhibitor protein has a Mr of circa 48,000 which would be encoded by a mRNA species of

FIGURE 3.24 DETERMINATION OF THE SIZE OF INSERTS  
SUBCLONED INTO pTZ

The cDNA inserts subcloned into the EcoR1 site of pTZ were released by digestion with EcoR1 (Section 2.13.1) and the digested products subjected to agarose gel electrophoresis (Section 2.9.7).

lane 2: 500ng uncut pTZ;

lane 3: 1000ng pRLRI 7;

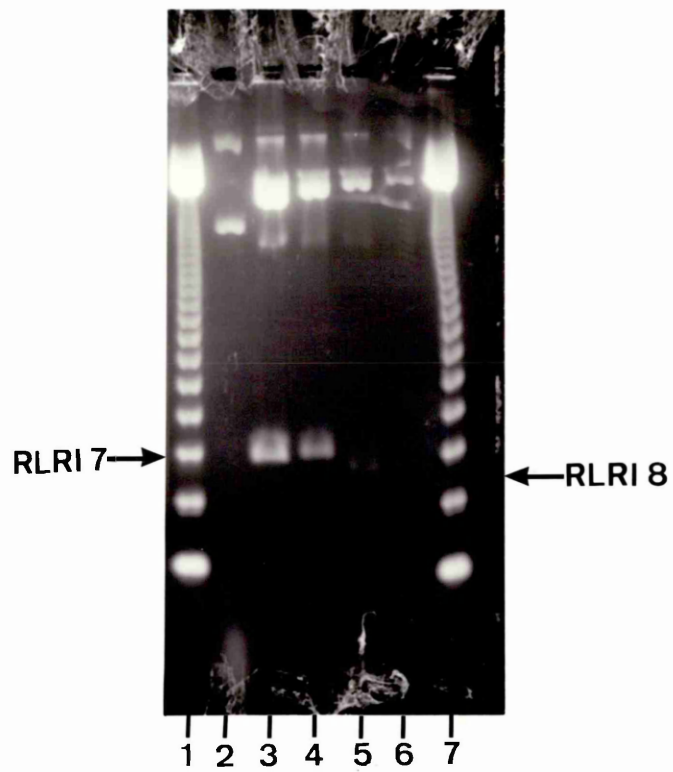
lane 4: 500ng pRLRI 7;

lane 5: 1000ng pRLRI 8;

lane 6: 500ng pRLRI 8;

lanes 1 and 7: 123bp DNA ladder.



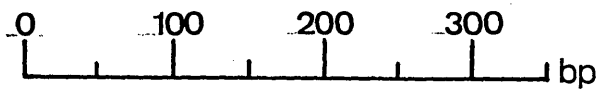


approximately 1.5-2.0 Kbp. Thus, the clones isolated from the rat liver library have the potential of encoding circa 100 amino acids which is approximately 20% of the total protein.

After ascertaining that the inserts had been successfully subcloned, recombinant plasmid DNA was used to transform *E.coli* MV1190. Transformed colonies were used to prepare single stranded DNA (Section 2.14.2) and T-tracking was carried out to determine the orientation of the subclones. The insert has the potential to be subcloned into the vector in one of two orientations and it is desirable to have the clones in both orientations so that sequencing of both strands can be carried out. Analysis of approximately 50 subclones revealed that all were in the same orientation (data not shown). Thus, an alternative method of subcloning was utilised to obtain subclones in the opposite orientation. The inserts from pRLRI 7 and pRLRI 8 were released from the plasmids by digestion with EcoR1 and purified from a low melting point agarose gel as described in Section 2.16.1. A known amount of insert (50ng) was ligated to a 20ng of vector DNA (which had also been digested with EcoR1) as described in the Amersham Cloning Manual and Section 2.13.3 and transformed into MV1190. Positive colonies were again taken through two rounds of purification, plasmid DNA isolated and digested with EcoR1 to ensure that inserts were present. Again single stranded DNA was prepared and the subclones analysed by T-tracking which revealed that two subclones of pRLRI 8 were in the opposite orientation compared to the others. A turn-around clone of pRLRI 7 was not obtained.

FIGURE 3.25 STRATEGY FOR SEQUENCING pRLRI 7 AND  
pRLRI 8

Subclones of pRLRI 7 and pRLRI 8 were obtained as described in the text (Section 3.5.), single stranded DNA prepared and the DNA sequenced by the Sanger dideoxy chain termination method (Section 2.14). The samples were electrophoresed for varying lengths of time (between 1 to 3 hours) to allow sequencing of different regions of the DNA.



RLRI 7

RLRI 7

RLRI 8

RLRI 8

RLRI 8

RLRI 8

RLRI 8

### 3.5.2 Nucleotide sequence analysis of rat liver cDNA clones

Sequence analysis was carried out on the different subclones by the dideoxy chain termination method as described in Section 2.14. As a control, non recombinant pTZ DNA was also sequenced to ensure that the sequence obtained from the subclones was not derived from the plasmid (data not shown). Figure 3.25 illustrates the strategy used to determine the sequence of the subclones.

A total of 177 bases were sequenced using the single stranded template from pRLRI 8 in one direction and 198 bases were sequenced from pRLRI 8 in the opposite direction. An overlap of 92 bases was observed and the full length of the insert from pRLRI 8 was determined to be 283 bases. The size of the insert from pRLRI 8 had previously been calculated to be 316bp (Section 3.5.1).

190 nucleotides were sequenced from subclone pRLRI 7 on the one strand only (as a turn around clone was not obtained). The first 65 nucleotides were not present on the sequence of pRLRI 8, but the remaining 125 nucleotides were identical to that from pRLRI 8. In Section 3.5.1, the size of the insert from pRLRI 7 was calculated from the agarose gel and found to be 371bp and that from pRLRI 8 was found to be 316bp i.e. a difference of 55bp. This compares well with the result presented here which suggests that the insert from pRLRI 7 is actually 65 nucleotides larger than that from pRLRI 8.

A compilation of the DNA sequence obtained from the inserts of pRLRI 7 and pRLRI 8 is shown in Figure 3.26. and the arrow

FIGURE 3.26 COMBINED DNA SEQUENCE OF pRLRI 7 AND pRLRI 8

Sequencing was performed as described in Section 2.14.

The DNA sequence of pRLRI 7 starts at nucleotide 1 and continues to nucleotide 190.

The DNA sequence of pRLRI 8 commences at position 65 (marked by the arrow) and continues to nucleotide 350.

Between nucleotide 65 and 190 inclusive, the sequence of both pRLRI 7 and pRLRI 8 were identical.

10 20 30 40 50  
GCGGCACTAT GGAAGACATG GCTTTTGCAG AGCGATTTGT TGTTGAGGCA  
CGCCGTGATA CCTTCTGTAC CGAAAACGTC TCGCTAAACA ACAACTCCGT

60 70 80 90 100  
GAAAGCTCT TGGGTGGACT GGACATGCTC ATTCTCAATC ACATCACACA  
CCTTTOGAGA ACCCACCTGA OCTGTAOGAG TAAGAGTTAG TGTAGTGTGT

110 120 130 140 150  
CGACCACTAT GTCTCTCTTC CAOGATGATA TCCACTCTGT GCGAAGAAGC  
GCTGGTGATA CAGAGAGAAG GTGCTACTAT AGGTGAGACA CGCTTCTTCG

160 170 180 190 200  
ATGGAGGTCA ACTTCCCTCAG CTATGTGGTC CTGAGCACAG CAGCCTGCC  
TACCTCCAGT TGAAGGAGTC GATACACCAG GACTCGTGTC GTCGGACGGG

210 220 230 240 250  
ATGCTGAAAC AGAGCAATGG CAGCATGGCC ATCATCTCCT CCATGGCTGG  
TACGACTTTG TCTCGTTACC GTCGTACCGG TAGTAGAGGA GGTACCGACC

260 270 280 290 300  
GAAAATGACC CAACCTCTGA TGCTCCTACT CTGCAAGGCA AGCTCTGGCT  
CTTTTACTGG GTTGGAGACT ACGAGGATGA GACGTTCCGT TCGAGACOGA

310 320 330 340 350  
CTGGATGGGT TCTTTTCCAC CATTAGAAAA GAACACTTGA TGACCAAGG  
GACCTACCCA AGAAAAGGTG GTAATCTTTT CTTGTGAACT ACTGGTTCC

marks the beginning of the sequence of pRLRI 8.

The open reading frames encoded by the nucleotide sequence were obtained using the MAP programme of the UWGCG package (University of Wisconsin Genetics Computer Group) on a VAX computer terminal in this department. As the cDNA is read in a 5' to 3' direction and it is unknown which is the coding strand, there are 6 possible reading frames as shown in Figure 3.27. None of these reading frames appeared to be similar to the amino acid sequence obtained for porcine liver and human placenta *Rnase inhibitor* (Hofsteenge *et al.*, 1988; Lee *et al.*, 1988).

At this time, the sequences of the human ribonuclease inhibitor (Lee *et al.*, 1988; Schneider *et al.*, 1988) had not been entered into either the GENBANK or the DATABANK libraries. Thus a direct comparison of the rat liver DNA sequence and the human ribonuclease inhibitor sequence was made using the programmes BESTFIT and GAP. The former programme revealed that there was a 77% homology over 52 bases as shown below:

```
1CGGCTGCGGGGTCCTGTCCAGCACACTACGCACCCTGCCCA
2CAGCTATGTGGTCCTG....AGCACA.....GCAGCCTGCCCA
```

```
1 CCCTGCAGGAG
```

```
2 TGCTGAAACAG
```

Sequence 1 corresponds to nucleotides 421 to 472 of the human placenta DNA sequence whereas sequence 2 corresponds to nucleotides 168 to 212 of the rat liver DNA sequence.

The data from GAP revealed that the two DNA sequences were unrelated.

The rat liver DNA sequence was then used to search the



FIGURE 3.27 PREDICTED AMINO ACID SEQUENCES FROM THE  
DNA SEQUENCE OF pRLRI 7 AND pRLRI 8

The six amino acid sequences predicted from the DNA sequence of pRLRI 7 and pRLRI 8 are shown. The amino acid sequences were determined using the MAP programme of the UWGCG package on a VAX computer.

The symbol \* indicates a stop codon.

1

60

GOGGCACTATGGAAGACATGGCTTTTGCAGAGOGATTTGTTGTTGAGGCAGGAAAGCTCT  
 CGOOGTGATAOCTTCTGTACCGAAAACGTCTCGCTAAACAACAACCTCOGTCOCTTTTCGAGA  
 A A L W K T W L L Q S D L L L R Q E S S  
 R H Y G R H G F C R A I C C \* G R K A L  
 G T M E D M A F A E R F V V E A G K L L

-----  
 R C \* P L C P K Q L A I Q Q Q P L F A R  
 P V I S S M A K A S R N T T S A P F S K  
 A S H F V H S K C L S K N N L C S L E Q

61

120

TGGGTGGACTGGACATGCTCATTCTCAATCACATCACACACGAOCCACTATGTCTCTCTTC  
 ACCCAOCTGAOCTGTACGAGTAAGAGTTAGTGTAGTGTGTGCTGGTGATACAGAGAGAAG  
 W V D W T C S F S I T S H T T T M S L F  
 G W T G H A H S Q S H H T R P L C L S S  
 G G L D M L I L N H I T H D H Y V S L P

-----  
 P H V P C A \* E \* D C \* V R G S H R E E  
 P P S S M S M R L \* \* M V C S W \* T E R G  
 T S Q V H E N E I V D C V V V I D R K W

121

180

CAOGATGATATCCACTCTGTGOGAAGAAGCATGGAGGTCAACTTCOCTCAGCTATGTGGTC  
 GAGCTACTATAGGTGAGACACGCTTCTTCGTACCTCCAGTTGAAGGAGTGGATACAOCCAG  
 H D D H I S V R R S M E V N F L S Y V V  
 T M I S T L C E E A W R S T S S A M W S  
 R \* Y P L C A K K H G G Q L P Q L C G P

-----  
 V I I D V R H S S A H L D V E E A I H D  
 R H Y G S Q A F F C P P \* S G \* S H P G  
 S S I W E T R L L M S T L K R L \* T T R

181

240

CTGAGCACAGCAGCOCTGCCATGCTGAAACAGAGCAATGGCAGCATGGCCATCATCTOCT  
GACTCGTGTGTCGGACGGGTACGACTTTGTCTCGTTACCGTCGTACCGGTAGTACACCA  
L S T A A C P C \* N R A M A A W P S S P  
\* A Q Q P A H A E T E Q W Q H G H H L L  
E H S S L P M L K Q S N G S M A I I S S

-----  
Q A C C G A W A S V S C H C C P W \* R R  
S C L L R G M S F C L L P L M A M M E E  
L V A A Q G H Q F L A I A A H G D D G G

241

300

CCATGGCTGGGAAAATGACCCAAOCTCTGATGCTOCTACTCTGCAAGGCAAGCTCTGGCT  
GGTACCGACCOCTTTTACTGGGTTGGAGACTACGAGGATGAGAOGTTCOGTTGAGACOGA  
P W L G K \* P N L \* C S Y S A R Q A L A  
H G W E N D P T S D A P T L Q G K L W L  
M A G K M T Q P L M L L L C K A S S G S

-----  
W P Q S F S G V E S A G V R C P L S Q S  
M A P F I V W G R I S R S Q L A L E P E  
H S P F H G L R Q H E \* E A L C A R A R

301

350

CTGGATGGGTTCTTTTCCACCATTAGAAAAGAACACTTGATGACCAAGG  
GACCTACCCAAGAAAAGGTGGTAATCTTTTCTTGTGAACTACTGGTTCC  
L D G F F S T I R K E H L M T K  
W M G S F P P L E K N T \* \* P  
G W V L F H H \* K R T L D D Q

-----  
Q I P E K G G N S F F V Q H G L  
P H T R K W W \* F L V S S S W  
S P N K E V M L F S C K I V L

GENBANK library to determine if the rat liver clones were related to any other gene which has been sequenced. It was found that the rat liver clones showed no homology to any other gene.

### 3.6 FURTHER CHARACTERISATION OF THE RAT LIVER cDNA CLONES

#### 3.6.1 Comparison of the rat liver cDNA clones with human placental ribonuclease inhibitor cDNA by Southern blot analysis

In the latter stages of this project, Lee *et al.*, (1988) reported the isolation of a human ribonuclease inhibitor clone from a cDNA library in  $\lambda$ gt11 using an antibody raised against placental ribonuclease inhibitor. The authors also reported the complete nucleotide and amino acid sequence of the human placental ribonuclease inhibitor (abbreviated to HPI) and Professor B.L.Vallee kindly gave some of this human clone to this laboratory. As it was still uncertain that the clones pRLRI 7 and pRLRI 8 encoded the ribonuclease inhibitor, it was decided to perform a Southern blot (Section 2.17.4) to determine if these clones cross-hybridised with the human clone.

It can be seen in Figures 3.28.A, B and C that pRLRI 7 hybridised to itself and to the other putative rat inhibitor clone, pRLRI 8, but it did not cross-hybridise to the human clone. Similarly, the human clone, pHPI, hybridised to itself but did not cross-hybridise to either of the rat clones. (A mouse actin cDNA, included as a control, hybridised to itself but to none of the other clones).

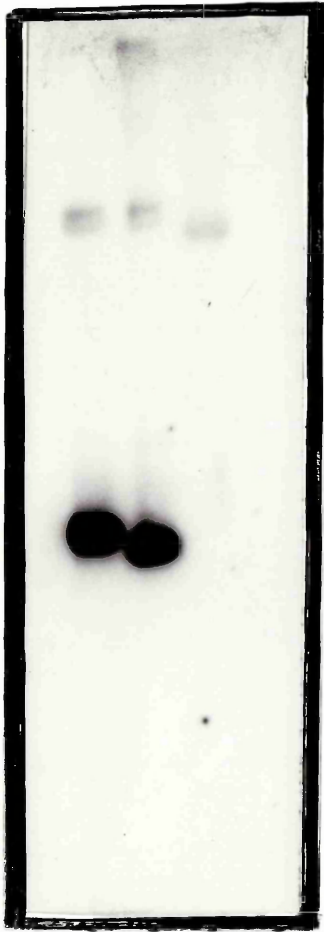
All three probes have hybridised to the higher molecular weight plasmid DNA. This was assumed to be a non-specific interaction due to the low stringency wash of the filters.

FIGURE 3.28 COMPARISON OF RAT LIVER CLONE, pRLRI 7 WITH HUMAN PLACENTAL INHIBITOR CLONE, pHPI BY SOUTHERN BLOTTING ANALYSIS.

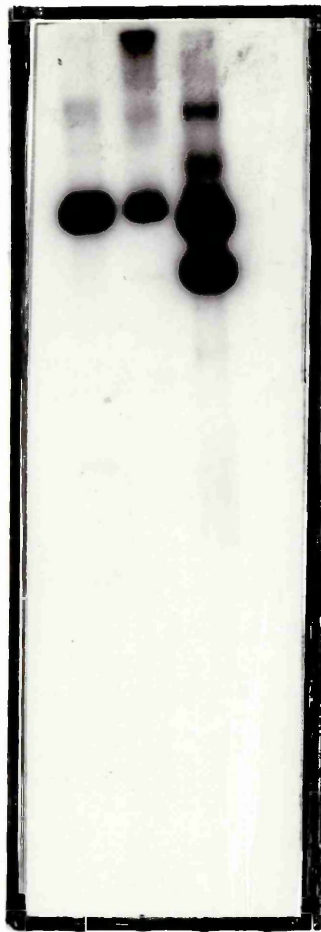
The putative rat inhibitor clones, pRLRI 7 and pRLRI 8 and the human inhibitor clone, pHPI were digested with EcoR1 to cut out the inserts (Section 2.13.1) and subjected to electrophoresis on a 1% (w/v) agarose gel (Section 2.9.7). Mouse actin cDNA was included as a control (Section 2.17.3). The DNA was transferred to Hybond N (Section 2.17.2) and the filter probed with  $^{32}\text{P}$ -labelled inserts (Section 2.16.2) from pRLRI 7 (Figure A), pHPI (Figure B) and mouse actin (Figure C). The Southern blots were washed with low stringency (2xSSC, 0.1% (w/v) SDS) at 60°C for 2x15 minutes and exposed for one hour.

In each of the three figures, A, B and C, lanes 1 to 4 are as follows:  
lane 1: 400ng EcoR1 digested pRLRI 7 (which provides 40ng insert);  
lane 2: 400ng EcoR1 digested pRLRI 8 (40ng of insert)  
lane 3: 160ng EcoR1 digested pHPI (40ng of insert)  
lane 4: 40ng of mouse actin cDNA insert.

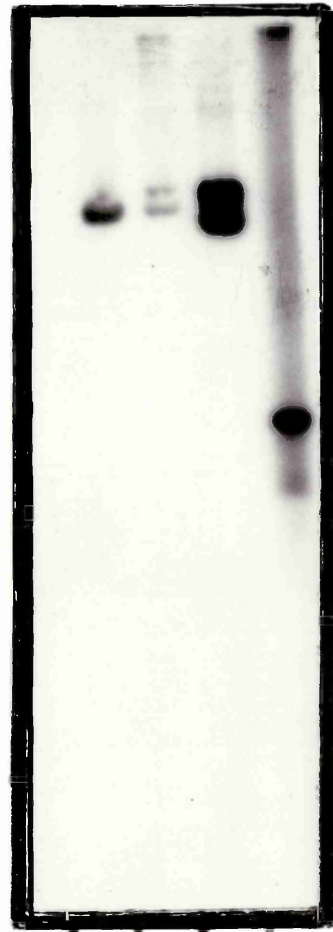
A



B



C



### 3.6.2 Comparison of the rat liver cDNA clones with the human placental ribonuclease inhibitor cDNA clone by northern blot analysis

There were two possible explanations of the data illustrated in the previous experiment (Figure 3.28). Either the rat and human ribonuclease inhibitors are so different that they do not cross-hybridise or the rat clones are not of the inhibitor. In an attempt to resolve these possibilities, northern blot analyses (described in Section 2.17.1) were performed to ascertain whether rat liver contained any mRNA species that would cross-react with the human probe. It can be seen in Figure 3.29 that while the rat and actin probes hybridised to the rat RNA (lanes 1 and 3 respectively), no cross-hybridisation was detected between the human clone and the rat RNA thus suggesting that the first of the above alternatives is correct. The rat probe hybridised to RNA of approximately 1.7 Kb which compares well with a reported size for the human mRNA of 1.9 Kb (Lee *et al.*, 1988; Schneider *et al.*, 1988). The actin probe hybridised to a mRNA species of 2.4 Kb.

Similar northern blots were attempted with RNA purified from human brain (data not shown) to determine if the rat clone hybridised to a mRNA species in human. However, none of the probes hybridised and it was assumed that the RNA had probably degraded.



FIGURE 3.29 NORTHERN BLOTTING ANALYSIS OF RAT LIVER  
RNA WITH THE RAT AND HUMAN cDNA CLONES.

Rat liver RNA (prepared as described in Section 2.15.1) was subjected to northern blot analysis as described in Section 2.17.1. As heterologous probing was taking place, the filters were washed with low stringency (2xSSC, 0.1% (w/v) SDS) at 60°C for 2x15 minutes.

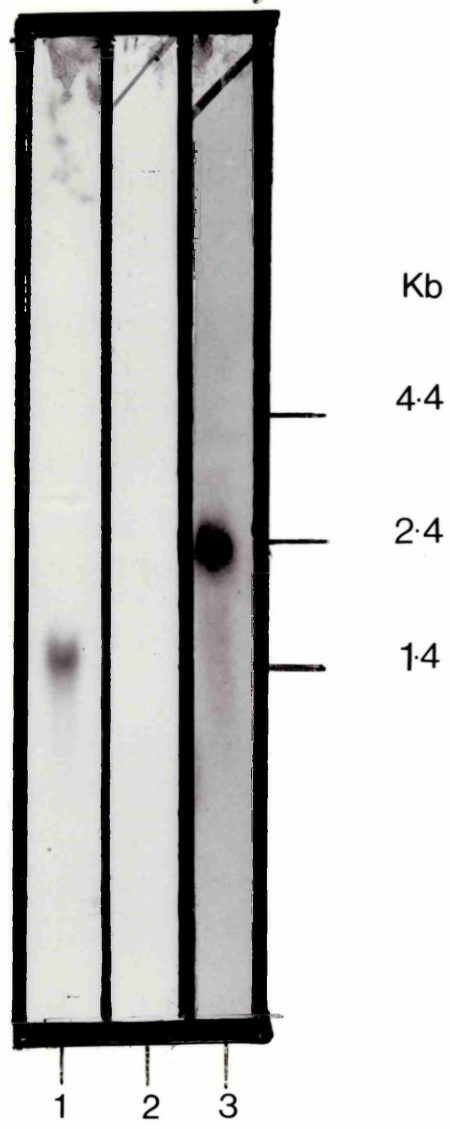
10µg of total rat liver RNA was present in each of the lanes and probed with the following:

lane 1:  $^{32}\text{P}$ -labelled insert from pRLRI 7;

lane 2:  $^{32}\text{P}$ -labelled insert from pHPI;

As no hybridisation was apparent on lane 2, this filter was stripped and re-probed with  $^{32}\text{P}$ -labelled mouse actin cDNA (result shown in lane 3).

RNA markers were run simultaneously and transferred to Hybond N. This lane was removed and the RNA stained with methylene blue as described (Section 2.17.1). The stained markers were aligned to the autoradiograms and marked as shown. The size of the transcripts recognised by the probes was determined as described in Section 2.17.2



### 3.6.3 Expression of the rat liver cDNA clones in rat tissues

It was hoped during the course of this project to confirm the identity of the isolated clones as rat ribonuclease inhibitor cDNA and to then use them to study the expression of the gene encoding the protein. It was planned to compare expression in different rat tissues and to then concentrate on the liver and look at expression in foetal versus adult tissue, after adrenalectomy and glucocorticoid treatment, after partial hepatectomy and in hepatoma. In the event, difficulties in confirming the identification of the recombinants resulted in none of this work being carried out except an initial analysis of different rat tissues.

Total RNA was isolated from the brain, heart, kidney and liver of rat by the method of Chirgwin *et al.*, (1979) as described in Materials and Methods (Section 2.15.1). 15µg of RNA from the different tissues was subjected to northern blot analysis as described in Section 2.17.1. The filter was probed with the insert from pRLRI 7 labelled with <sup>32</sup>P by the random priming method (Section 2.16.2).

Figure 3.30.A shows that there appears to be a high level of expression of sequences complementary to the rat liver clone in rat kidney (lane 3) and liver (lane 4) and a lower level of expression in rat brain (lane 1) and heart (lane 2). To determine if this pattern of expression is representative or is merely due to incorrect loading of the RNA, the filter was stripped and re-probed with an actin cDNA. Approximately equal levels of actin mRNA would be expected in all four tissues. As seen in Figure 3.30.B, there was an unequal

**FIGURE 3.30 TISSUE EXPRESSION STUDIES USING THE RAT LIVER cDNA CLONE, pRLRI 7.**

RNA isolated from rat brain, heart, kidney and liver (Section 2.15.1) was probed with  $^{32}\text{P}$ -labelled insert from pRLRI 7 (Figure A) as described in Section 2.17.1. The filter was washed with high stringency (0.1XSSC, 0.1% (w/v) SDS) at 60°C for 2x15 minutes and exposed for 3 days. The filter was then stripped and reprobbed with mouse actin cDNA (Figure B) and processed as above.

In both Figures A and B, each lane contains 15µg of total RNA from different tissues as follows:

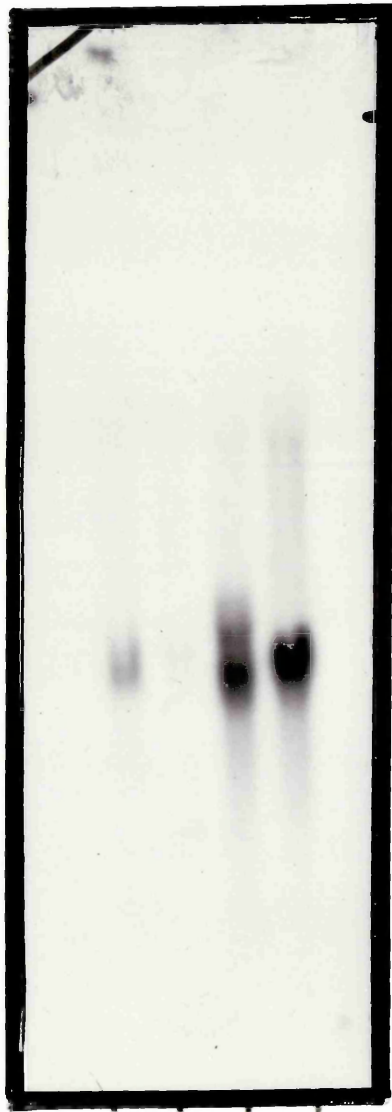
lane 1: rat brain;

lane 2: rat heart;

lane 3: rat kidney;

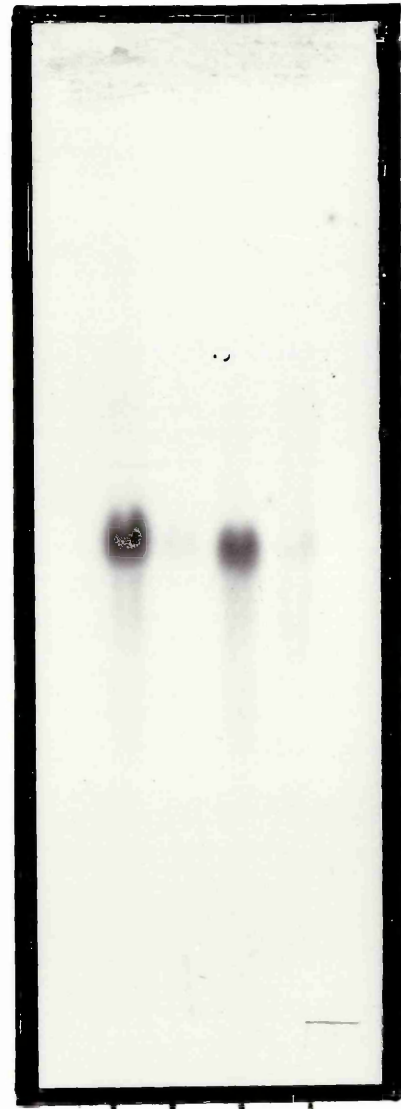
lane 4: rat liver.

A



1 2 3 4

B



Kb

4.4

2.4

1.4

1 2 3 4

loading of RNA i.e. the amount of heart (lane 2) and liver (lane 4) RNA loaded onto the gel were comparable but were several orders of magnitude lower than that of brain (lane 1) and kidney (lane 3). Notwithstanding the inadequacies of this experiment, which there was unfortunately no time to repeat, some features are noteworthy. It is clear that the expression of the cloned species in brain is less than that in either liver or kidney and there is a very high level of expression of the clone in rat liver. Also, the clone appears to hybridise to two species of RNA in the kidney.

**CHAPTER FOUR**

**DISCUSSION**

The aim of this project was to obtain a cDNA clone of the ribonuclease inhibitor to use as a probe in determining the extent to which inhibitor activities are controlled at the level of gene expression. It was decided to work with the rat liver system as it is an organ with a high level of protein synthesis and a high level of inhibitor activity and was therefore, an ideal source of this protein (Shortman, 1962; Roth, 1962). In addition, several studies have been carried out on the rat liver and it has been shown that changes occur in the level of inhibitor activity depending upon the metabolic state of the liver e.g. in the regenerating rat liver (Moriyama *et al.*, 1969) and in the liver of adrenalectomised rat (Lui and Matrisian, 1977).

The initial task in this project was to purify ribonuclease inhibitor and use the purified protein to obtain a highly specific polyclonal antibody with which to screen a rat liver  $\lambda$ gt11 expression library. Thus, the ribonuclease inhibitor was routinely prepared from rat liver by the method of Burton (1980) with the modification that the protein was eluted at 4°C rather than 25°C. In general, the specific activity was approximately  $1.1 \times 10^5$  units/mg and a typical yield was 40%. These results are similar to those obtained by N. Brockdorff, a previous member of this laboratory who also routinely prepared inhibitor from rat liver. In this study, the inhibitor was purified approximately 8,500-fold which is in agreement with Yasuna and Goto (1986) who obtained a purification factor of 10,000-fold. This is in contrast to that of Brockdorff and Knowler (1986) who reported a purification factor of 26,000-fold. The reason for the discrepancy in these results was



unclear. The relative molecular mass (Mr) of the rat liver inhibitor was calculated to be approximately 46,700 Daltons by SDS PAGE which is in agreement with that of Burton and Fucci (1982) who obtained a Mr of 48,600

Analysis of the purified protein by SDS-PAGE revealed that the preparation contained small amounts of contaminating proteins, a result similar to that reported by Burton and Fucci (1982) and Brockdorff (PhD thesis, 1985). They overcame this problem by subjecting the purified inhibitor to the affinity chromatography step again which apparently yielded more homogenous preparations of protein.

Brockdorff then utilised this homogenous preparation of ribonuclease inhibitor to raise polyclonal antiserum in rabbits. Analysis of this antiserum by western blotting revealed that it contained antibodies which recognised other proteins from rat liver extracts in addition to ribonuclease inhibitor. The presence of these contaminating antibodies was attributed to trace amounts of protein thought to be present in the injected protein preparation, despite rechromatography on the RNase A-Sepharose affinity column. Therefore, in this study it was decided to apply the non homogenous protein preparation obtained after the affinity chromatography stage to a preparative SDS polyacrylamide gel to resolve the contaminating proteins from the inhibitor. The band corresponding to the inhibitor was excised from the gel and used in immunisation. A similar method for purifying antigen was utilised by A. Hunter (PhD thesis, 1985) who isolated the individual subunits of mammalian 2-oxo-glutarate dehydrogenase complex and

used them to raise polyclonal antiserum.

Despite these precautions, when the anti-ribonuclease inhibitor antiserum was used to challenge a cytoplasmic liver extract on a western blot, a similar result was obtained to that of Brockdorff (PhD thesis, 1985) i.e. other immune bands were apparent in addition to the band corresponding to the inhibitor, thus indicating the presence of additional antibodies in the immune serum. In this project, these additional antibodies were also present in pre-immune serum (i.e. serum obtained from the rabbit prior to the initial injection of the antigen) which suggested that the presence of these contaminating antibodies was not due to impurities present in the injected inhibitor preparation. The antibodies which were raised against ribonuclease inhibitor were separated from the additional antibodies by 'negative' affinity chromatography. The immune serum was passed through a bed of Sepharose 4B coupled to a rat liver protein preparation which was depleted in ribonuclease inhibitor. Thus, antibodies raised against the inhibitor did not bind to the column as no antigen was present. Antibodies recognising other liver proteins bound to their respective antigens on the column and were absorbed from the immune serum. The absorbed antiserum was now considered to be monospecific as it now recognised only one protein (ribonuclease inhibitor) on a western blot of a rat liver extract.

An alternative method for purifying specific antibodies is to bind the antigen to a Sepharose column. The specific antibodies bind to the antigen and can be eluted using ammonium hydroxide or glycine pH2 (Johnstone and Thorpe 1982). This was the preferred method of

many researchers who have screened  $\lambda$ gt11 expression libraries with polyclonal antibodies (Ny *et al.*, 1986; Saris *et al.*, 1986; Tamkun *et al.*, 1986; Jahnsen *et al.*, 1986; Nagata *et al.*, 1986 and Ogawa *et al.*, 1988).

It was of vital importance to obtain a high titre, monospecific polyclonal antibody for screening the cDNA expression library. If the antiserum recognised more than one protein from a crude cell liver extract on a western blot, then there may be a chance that the serum would recognise these additional proteins in a screen of the library. Thus, there would be an uncertainty that the cDNA of interest had been cloned. Monoclonal antibodies have been used to isolate genes (Ishida *et al.*, 1987) but they are not as ideal for screening an expression library as polyclonal antibodies. In general, a monoclonal antibody recognises a single epitope of a protein. Thus, if the cDNA was incomplete i.e. did not encode the entire protein and that part of the protein which contained the epitope was absent, the antibody would not recognise this clone. Also, it is possible that an entirely different protein may contain a similar epitope and this may be recognised by the monoclonal antibody. Thus, there is an increased chance that false positives may be obtained when using monoclonal antibodies.

When screening an expression library, it is advantageous to obtain a high signal to noise ratio which facilitates in the detection of antigen producing plaques from the background obtained from non-antigen expressing plaques. Several steps were taken to reduce background levels; IgG was purified from immune serum by ion-exchange chromatography and the IgG preparation was adsorbed

with a bacterial lysate of the host strain of bacteria to remove any antibodies which bound to *E.coli* antigens (Helfman *et al.*, 1985; Huynh *et al.*, 1985).

Thus, a monospecific, polyclonal antibody was prepared which produced a strong signal on a western blot. However, before the library was screened, the best method for detecting bound antibody was determined. Essentially, there were three options:  $^{125}\text{I}$ -labelled protein A (prepared by J.A. Hodgson, this department); horseradish peroxidase (HRP) conjugated secondary antibody (SAPU) and biotinylated secondary antibody (Amersham). Dot blot analysis of purified ribonuclease inhibitor was carried out using each of the aforementioned methods to detect bound antibody. It was found that the two latter methods were comparable and at least ten times more sensitive than the  $^{125}\text{I}$ -labelled protein A method. These results were different to those reported by Young and Davis (1985) who found that  $^{125}\text{I}$  protein A was comparable to the biotinylated secondary antibody and both were reported to be 5-10 times more sensitive than HRP conjugated secondary antibody. The result obtained using  $^{125}\text{I}$ -labelled protein A in this project can be explained as it had a specific activity 10 times less than that used by Young and Davis (J.A. Hodgson, personal communication). As the other two methods provided similar results and as HRP secondary antibody is provided *gratis* to Scottish University research groups, it was decided to use this method to detect bound antibody.

A further point to arise from the dot blot analysis was that the antibody recognised native protein despite being raised against denatured protein. This was important as the fusion proteins which

are produced when screening the library are in the native state and the antibody must be able to recognise them in this form. It was also important that, when using the selected method, it was possible to detect 1.0ng of purified ribonuclease inhibitor. The antigen-producing plaques may not produce large quantities of fusion protein and therefore the antibody must be able to detect small amounts of antigen.

The library which was used in this study was donated by R.O. Hynes in 1984 and its preparation is described by Schwarzbauer *et al.*, (1983). The authors of this paper reported that the library contained 85-90% recombinant phage whereas in this study, the number of recombinants from the same library was found to be 56%. As the library was stored for several years before use, it was possible that unstable recombinant phage had spontaneously ejected their inserts.

After three successive rounds of screening, three recombinant clones were isolated from the library and subsequently named  $\lambda$ RLRI 3,  $\lambda$ RLRI 7 and  $\lambda$ RLRI 8. At the onset of the project, it was hoped to screen the library with two separate antisera raised against ribonuclease inhibitor i.e. to take duplicate filters of the plated phage, screen with the two antibodies and choose the positives common to both antibody preparations. It was thought that screening the library with antiserum from two independent sources would decrease the probability of isolating false positives. However, the antiserum produced by the second rabbit did not have a workable titre, nor did it detect less than 10ng of purified ribonuclease inhibitor in dot blot analysis. It was therefore

considered to be unsuitable for screening the library.

Lysogens were prepared from the three isolated recombinant phages for several reasons. Firstly, the phage DNA was more stable when integrated into the bacterial chromosome and secondly, preparative amounts of fusion protein can be produced for further characterisation (Huynh *et al.*, 1985)

Analysis of the fusion proteins produced by  $\lambda$ RLRI 3 and  $\lambda$ RLRI 8 revealed that they were IPTG-inducible and had an approximate relative molecular mass (Mr) of 127,000 Daltons. As the Mr of the  $\beta$  galactosidase portion of the fusion protein was 114,000 Da (Young and Davis, 1985), the polypeptide encoded by the cDNA was approximately 13,000 Daltons. In this particular experiment, it was noted that the fusion protein produced by the  $\lambda$ RLRI 7 lysogen was expressed constitutively i.e. it was not IPTG-inducible. This may have been due to the lambda repressor having been titrated out of this bacterial population. This result was not reproducible as other preparations of fusion protein from this lysogen were inducible with IPTG. The fusion protein for this lysogen had an approximate Mr of 130,000 Daltons. As the Mr of rat liver ribonuclease inhibitor was approximately 48,000 Daltons (Burton and Fucci, 1982), it was clear that none of the three clones isolated from the library encoded the entire inhibitor protein.

The isolated clones were further characterised by western blot analysis of the uninduced and IPTG-induced bacterial lysate of the three recombinant lysogens and the non recombinant lysogen,  $\lambda$ gt11/Y1089. This revealed that the fusion proteins from the lysogens of  $\lambda$ RLRI 7 and  $\lambda$ RLRI 8 produced an immunopositive

fusion protein whereas an immune band was not observed for  $\lambda$ RLRI 3 in Y1089. The IPTG-induced bacterial lysate of  $\lambda$ gt11/Y1089 contained non recombinant  $\beta$ -galactosidase and this was included in the western blot as a control to ensure that the antibody was recognising the protein encoded by the cDNA insert and not the  $\beta$ -galactosidase portion of the fusion protein. Thus,  $\lambda$ RLRI 7 and  $\lambda$ RLRI 8 appeared to contain ribonuclease inhibitor antigenic determinants within their respective fusion proteins. Interestingly, detection of the fusion proteins required the biotinylated secondary antibody detection system (which is a reputedly 10 times more sensitive than the HRP secondary antibody system) before immune positive bands were observed. This was probably due to  $\beta$ -galactosidase antigenic determinants masking the antigenic sites of the smaller cDNA-encoded polypeptide. This hypothesis was corroborated by the results obtained from the immunogenic peptide maps which were developed using the HRP secondary antibody system i.e. digestion of the fusion proteins revealed the antigenic sites of the cDNA-encoded polypeptide.

Phage DNA was isolated from the recombinant lysogens  $\lambda$ RLRI 7/Y1089 and  $\lambda$ RLRI 8/Y1089 and, after digestion with EcoR1 to release the insert, it was found that the insert from  $\lambda$ RLRI 7 was 371bp and that from  $\lambda$ RLRI 8 was 316bp. Thus insert 7 was 55bp larger than that for insert 8. This was expected as the fusion protein from  $\lambda$ RLRI 7 was larger than that of  $\lambda$ RLRI 8. Also, 371bp is sufficient to encode a polypeptide of approximately 15,000 Daltons and 316bp has the potential of encoding for a polypeptide of 13,000 Daltons. These figures are very similar to

the Mr values obtained for the cDNA-encoded portion of the fusion protein from  $\lambda$ RLRI 7 (16,000 Daltons) and  $\lambda$ RLRI 8 (13,000 Daltons)

The library used in this project has also been used to isolate fibronectin (Schwarzbauer *et al.*, 1983) and UDP-glucuronyl transferases (Burchell *et al.*, 1984) and  $\alpha$ -inhibitor III (Aiello *et al.*, 1988). The initial clone isolated by Schwarzbauer and colleagues was 500bp and encoded a protein of 14,000 Daltons. The cDNA insert was sequenced and the predicted amino acid sequence compared to that of bovine plasma fibronectin which confirmed that it was a fibronectin clone. The 500bp clone was used to re-screen the library by plaque hybridisation and other clones were isolated which ranged in size from 500 to 3,000bp. Burchell *et al.* isolated 4 clones coding for UDPGT ranging in size from 1500-2000bp which encoded proteins of up to 32,000 Daltons. The clone isolated by Aiello *et al.*, was 1,400bp in length. Thus, the clones isolated in this project were smaller than those isolated by other research groups using the same library.

Interestingly, the clones isolated by Schwarzbauer *et al.*, (1983) did not all encode the 3' terminal end of the protein despite the cDNA being primed by oligo(dT). Of the six clones isolated, only two encoded the 3' end and 2 encoded the 5' end. Thus it was possible that the partial clones isolated in this study could encode any part of the inhibitor protein and not be confined to the 3' terminus. This can arise by incomplete synthesis of the second cDNA strand.

The next stage of this project was to determine if the two clones,



$\lambda$ RLRI 7 and  $\lambda$ RLRI 8, encoded ribonuclease inhibitor. As there was no published amino acid or nucleotide sequence of the inhibitor from any source at this time, a novel method was attempted to compare peptide maps of purified rat liver ribonuclease inhibitor and fusion protein. However, the task of isolating the fusion protein from a crude cell lysate proved to be somewhat difficult. Initially, the methods of isolating the fusion protein were based upon attempting to extract the fusion protein from a polyacrylamide preparative gel. The method of Tolan *et al.*, (1980) which involved extracting the protein by diffusion yielded little or no protein. This method has been used successfully in this laboratory by Z. Seraj (PhD thesis, 1986) who eluted hnRNP core proteins from SDS polyacrylamide gels. Similarly, electroelution of the protein from the gel yielded only 1-2% of the initial protein. It was unclear as to why the fusion protein proved difficult to isolate using these methods but it was possible that the size and conformation of the protein was not conducive to these types of elution.

As mentioned in the Results chapter, (Section 3.3.5), there is evidence that in a majority of cases, genes expressed as fusion proteins in *E.coli* are present in an insoluble form known as 'inclusion bodies' e.g. insulin A and B chains (Goeddel *et al.*, 1979),  $\beta$ -endorphin (Shine *et al.*, 1980) and prochymosin (Marston *et al.*, 1984). These inclusion bodies can be isolated by a single centrifugation step. The fusion protein produced by IPTG-induction of  $\lambda$ RLRI 7/Y1089 and  $\lambda$ RLRI 8/Y1089 were soluble as they were present in the supernatant fraction after centrifugation. Attempts were made to render the fusion protein insoluble by changing the pH and ionic strength of the lysis buffer but, in all

cases, the fusion protein remained soluble. It should be noted that examination of the literature concerning fusion proteins produced by  $\lambda$ gt11 recombinant lysogens showed that, in all cases examined, the fusion protein was always present in a soluble form (Schwarzbauer *et al.*, 1983; Burchell *et al.*, 1984; Ny *et al.*, 1986; Evans *et al.*, 1986; Weil *et al.*, 1987).

In retrospect, it may have been advantageous to attempt to purify the fusion protein by classical purification methods e.g. gel filtration or affinity chromatography on p-aminophenyl b-D thiogalactoside-Sepharose (Ullmann, 1984).

When it became apparent that the fusion protein was not easily purified from a crude cell lysate by the aforementioned methods, it was decided to isolate both the fusion protein and the purified inhibitor as a protein band from a coomassie blue stained preparative gel and use the method of Cleveland *et al.*, (1977) in conjunction with western blotting to produce an immunopeptide map. Unfortunately, the resultant maps were difficult to interpret but there was some evidence that there were peptides common to both fusion protein and ribonuclease inhibitor after digestion with chymotrypsin.

When it became apparent that the combined methods of Cleveland mapping and western blotting were not aiding in the identification of the recombinant clones, it was decided to obtain as much amino acid sequence of ribonuclease inhibitor as possible and then to compare this with the amino acid sequence predicted from the DNA sequence of the clones.

Of the five peptides on which the sequencing reaction was

attempted, only one provided some sequence data. It was unclear as to why the other peptides did not yield data as it was shown that they were present in sufficient quantities to be sequenced. It was possible that some of the peptides were derived from the N-terminal end (which has since been shown to be blocked by Schneider *et al.*, 1988) and this prevented them from undergoing the sequencing reaction. However, it seemed unlikely that all four peptides were derived from the N-terminus. The procedure utilised in this study to obtain peptides of human ribonuclease inhibitor was very similar to that used by Hofsteenge *et al.*, 1988. Both procedures involved reducing and alkylating the protein, followed by digestion with protease and isolation of peptides using reverse phase chromatography on HPLC. The main difference between the methods was in the choice of proteases. In this study, the inhibitor was digested with either trypsin or chymotrypsin whereas Hofsteenge and colleagues used cyanogen bromide, trypsin and *S.aureus* V8 protease. Similar systems were also used to sequence the peptides, however, Hofsteenge *et al.*, obtained amino acid sequence of the entire protein whereas only 11 amino acids were obtained in this project.

When the above data became available, the amino acid sequence for the rat liver obtained from this project was compared to that of porcine liver (Hofsteenge *et al.*, 1988) and human placenta (Lee *et al.*, 1988). The short stretch of amino acid sequence from the rat liver corresponded to residues 20-31 in the human placental sequence and to residues 15-26 in the porcine sequence and are shown below:

rat liver:           L T E L G P L I Q Q Y G

porcine liver:   W T E L L P L L Q Q Y E

human placenta: W A E L L P L L Q Q C Q

The homology between residues 20-31 of human placenta and 15-26 of porcine liver was calculated to be 75%. This compared well with the homology of the total protein from pig and human which was found to be 77% (personal calculation) Thus, the ribonuclease inhibitor from different species appears to be homologous as predicted by the amino acid compositions (Burton, 1980; Burton and Fucci, 1982; Blackburn *et al.*, 1977; Fominaya *et al.*, 1988a). Furthermore, if the homology of the above stretch of amino acids was representative of that between the whole protein sequence, then it would be expected that ribonuclease inhibitor from rat and pig would show 66% homology while that from rat and human would be 50% homologous.

Burton and Fucci (1982) raised antiserum against human placental inhibitor which did not cross-react to a great extent with the ribonuclease inhibitors from the livers of five mammalian species, including pig. They proposed that the secondary structure of the human placental inhibitor may be different to that of mammalian liver, despite the similarity in amino acid compositions. However, recently Bloemendal and Jansen, (1988) used antibodies raised against human placental ribonuclease inhibitor to detect inhibitor from a wide variety of tissues and species using western blotting analysis which suggests that the secondary structures of the inhibitors are conserved.

Six translational reading frames were obtained from the cDNA

sequence of the clones, none of which appeared to encode an amino acid sequence similar to that of ribonuclease inhibitor from pig or human. However, it should be noted that it is difficult to be entirely sure of predicted amino acid sequences when the DNA has not been sequenced totally on both strands as insertion or deletion of a single nucleotide can alter the reading frame. There were several reasons as to why the clones were not sequenced fully on both strands: Firstly, as mentioned in Section 3.5.1, there was great difficulty in obtaining 'turn around' clones and secondly, when a turn around clone was obtained, the single-stranded DNA preparations were not of high quality which hindered the reading of the sequencing gels. Analysis of the predicted amino acid sequences obtained from the region of DNA which had been read on both strands should, in theory, provide the most accurate amino acid sequence. However, no homology was discernible between this region and the human or porcine amino acid sequences.

An alternative strategy to obtaining DNA sequence on both strands would have been to sequence as much as possible of one strand, then determine possible restriction sites within this strand and, using these restriction sites, generate smaller subclones which may have been easier to read. However, time did not allow for this to be carried out.

The lack of homology between the human and porcine inhibitors and the predicted amino acid sequences of the isolated clones extended to the DNA sequences themselves. The combined DNA sequence of the clones pRLRI 7 and pRLRI 8 was compared by computer to that of human placental inhibitor and no homology was

evident (except for a stretch of 52 nucleotides which showed 77% homology). This was not entirely unexpected as the human cDNA clone did not hybridise to the rat liver clones on a Southern blot.

Northern blotting analysis of rat RNA probed with the isolated rat clones showed that the corresponding mRNA was approximately 1.7Kb. This is similar in size to the RNA which encodes the human ribonuclease inhibitor (Schneider *et al.*,1988; Lee *et al.*,1988) who obtained a value of 1.9Kb. Thus, the RNA corresponding to the rat clones is of a sufficient size to encode a protein with a Mr similar to that of the inhibitor.

When rat liver RNA was probed with the human placental clone, no hybridisation was evident. This was a surprising result as the proteins from different species appear to exhibit a high degree of homology, and it would be expected that the homology at the nucleotide level would also be relatively high. It is possible that the mRNA encoding ribonuclease inhibitor is of very low abundance and if more RNA had been loaded, then a signal may have been detected.

The preliminary results on the tissue specificity were interesting in that the clones appeared to be expressed in all four tissues studied i.e. brain, heart, kidney and liver. This was the expected expression pattern for ribonuclease inhibitor as the protein has been found in almost all tissues (Blackburn and Moore,1982). Also, the gene was expressed to a higher level in the liver and kidney than in the brain. Again, this was the pattern expected for the ribonuclease inhibitor as it has been reported that the level of inhibitor protein in the brain was one fourteenth that of placenta (Burton,1980)

With the available evidence, it was difficult to decide if an

authentic rat liver ribonuclease inhibitor clone had been isolated. The clones reacted very strongly with the monospecific anti-ribonuclease inhibitor antibodies and produced an immunopositive fusion protein. Also, the mRNA which hybridised to the clones was of a size which was large enough to encode a protein the size of ribonuclease inhibitor. Furthermore, the gene appeared to be expressed in the expected manner of ribonuclease inhibitor. However, the clones did not hybridise to the human placental clone in Southern analysis, nor did the DNA sequences compare (except for one small stretch of nucleotides). In addition, the predicted amino acid sequence of the rat liver clones did not appear to be similar to those from human or porcine inhibitor (and it would be expected that at least 50% homology with human and 67% with pig would be apparent).

As the clones isolated from the rat liver library did not show any homology to the human inhibitor, the DNA sequence was used to search the GENBANK to ascertain if another rat liver protein had been cloned instead. Interestingly, this sequence of DNA did not appear to be homologous to any other rat liver gene which has been sequenced and entered into the GENBANK files. Also, the cDNA did not appear to be similar to any gene from other sources. Thus, the identity of the clones isolated from the rat liver library remains unknown.

#### What went wrong?

This is an extremely difficult question to answer. When an

expression library is screened, the quality of the antibody probe is of vital importance and it was thought that reasonable care was taken to ensure that the antibody was optimal. The antibody appeared to be monospecific as it reacted with a single protein from a cytoplasmic fraction of rat liver proteins. However, the antibody was not tested against a fraction containing membranes and it is possible that the antibody cross-reacted with a membrane-bound protein with a similar Mr to that of ribonuclease inhibitor.

One method for determining the identity of isolated clones using the antibody is to affinity purify the antibodies which bind to the fusion protein and then, using these antibodies, to perform western blot analysis on the protein of interest. If the affinity purified antibodies also recognise this protein, then it can be assumed that they share antigenic determinants. This procedure has been used successfully by several groups (Ny *et al.*, 1986 and Weil *et al.*, 1987).

An alternative approach to proving the identity of an isolated clone is to perform hybrid-release translation as suggested by Young and Davis, (1985), Brandis *et al.*, (1986) and Weiss *et al.*, (1986). Briefly, this involves binding the isolated clone to a membrane filter and incubating with poly(A)<sup>+</sup> RNA prepared from the tissue of interest. Unbound RNA is washed away, the bound poly(A)<sup>+</sup> eluted and translated in an *in vitro* system. The translated products are subjected to SDS page, blotted onto nitrocellulose and challenged with the antibody that was originally used to isolate the clone. In this project, this approach was not taken as, firstly it was known from northern blotting analysis that the mRNA



corresponding to the cDNA clone encoded a protein of approximately the correct molecular weight and secondly, if a positive result was obtained, this would only show that a gene which encoded a protein recognised by the antibody had been cloned. It would still be uncertain if that protein was ribonuclease inhibitor.

With the advantage of hindsight it is worth analysing whether the correct approach to this project was to attempt to clone the inhibitor protein from an expression library. The literature includes many testaments to the fact that this approach can be successful. Indeed, this is how the human placental inhibitor was recently cloned (Lee *et al.*, 1988). Nevertheless, discussion with colleagues and other scientists in a number of institutions reveal that the experience of this project, in cloning what are probably false positives from a  $\lambda$ gt11 library, is far from unique. A specific example is that reported by Boutin *et al.*, (1988) who attempted to isolate the prolactin receptor from a  $\lambda$ gt11 expression library using both polyclonal and monoclonal antibodies. They repeatedly isolated false positives as determined by northern blotting analysis. Eventually, these authors constructed oligonucleotides from the amino acid sequence and used these to isolate the required clones from the same library.

When trying to clone a protein for which no sequence is known, proof of the identity of isolated clones is likely to rest on sequencing both the cloned DNA and at least some of the protein. If then, some protein sequencing must be done, it can be argued that it is better done sooner than later so that the sequence generated can

be used to synthesise corresponding oligonucleotides. One could then screen a non-expressing cDNA library, such as  $\lambda$ gt10, and save the labour of raising and purifying antisera. However, many workers also record false positives after screening with oligonucleotides. This is especially true when only short stretches of protein sequence are known as there is then little flexibility in the nature of the oligonucleotides that can be made. Under these circumstances, it is unlikely that it will be possible to choose an oligo encoding a high percentage of amino acids with a low level of redundancy in their genetic code and oligos with a high level of redundancy are very likely to select false positives.

Perhaps, in the present case, more effort should have been put into sequencing earlier in the project so that oligonucleotides could be made. Recombinants selected by antibody screening of the  $\lambda$ gt11 library could then have been rapidly checked for cross-hybridisation to the oligonucleotides.

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