TITLE THE EXPRESSION OF BIOLOGICALLY ACTIVE RECOMBINANT RICIN A CHAIN *in vitro*.

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INSTITUTION and DATE University of Warwick 1988

THE EXPRESSION OF BIOLOGICALLY ACTIVE RECOMBINANT RICIN A CHAIN *in vitro*.

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A thesis submitted for the degree of

Doctor of Philosophy

at the University of Warwick

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U.K.

November, 1988.

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ACKNOWLEDGEMENTS

I would like to thank my supervisor, (Prof) Mike Lord for his guidance in this project. I would also like to express my thanks to all the people, not just in the ricin group who unselfishly provided technical expertise and helpful criticism of my work. In particular, I am grateful to Lynne Roberts for her advice, Martin Hartley, for his useful discussion and much appreciated help with ribosomal work, Debbie Gill and Saskia van der Vies for their expertise in deletion and plasmid sequencing work and Malcolm McCrae for his help and advice concerning mutagenesis. For his moral guidance, I am indebted to Gary Clements, whilst the less sensible influence of Jim Tregear is nonetheless much appreciated. Respect is due too, to Susan Jones, for her support when it was most needed.

Without the support of my sister, Alison, and her family, this work would not have been possible, I hope I will be able to repay their kindness and the endless opportunities they have presented in the years to come. Similarly, I am deeply grateful for the invaluable attention to my well being of Martin, Mike, June and Judy.

I acknowledge the financial support of the SERC Biotechnology directorate and Lynne Roberts and Colin Murrell, for their help in this respect too.

DECLARATION

All the results presented in this thesis were obtained by the author, apart from those which are specifically indicated in the text. All of the ricin treated ribosomal RNA analysis was performed by Martin Hartley and the deletion of the DNA sequence showing homology with hamster EF 2 was performed by Paul Krieg, figure 7.2.1.

All sources of information and materials have been specifically acknowledged in the text. None of the work contained in this thesis has been used for any previous application for a degree.

MMay

Michael May.

SUMMARY

The major aim of this project was to attempt to define residues in ricin A chain which are involved in the catalytic activity of the protein and to define a ricin A chain molecule of minimum size which still remains active.

A simple and sensitive system was developed in which the expression and assessment of biological activity of recombinant ricin A chain are combined. This represents one of few reported examples of the ability to assess the activity of protein expressed from in vitro synthesised RNA in a cell free system. When recombinant ricin A chain transcripts were translated in a rabbit reticulocyte lysate, the ribosomes were rapidly inactivated. In contrast, ribosomes which have translated transcripts encoding non toxic polypeptides such as ricin B chain are not inactivated. Ribosome inactivation was accompanied by a highly specific modification of 28S rRNA which is thought to cause the inactivation of the ribosomes. Protein synthesis by wheat germ ribosomes was not inhibited under conditions which inhibit reticulocyte ribosomes, confirming earlier observations that plant cytoplasmic ribosomes are much less sensitive to inactivation by ricin A chain than are mammalian ribosomes.

Using the same system, it was shown that by deleting an internal hexapeptide which shares homology with hamster EF 2, catalytic activity was completely abolished. Deleting a second internal pentapeptide, conserved between ricin A chain and trichosanthin, had no effect. Deleting the first nine residues from the N terminus of ricin A chain did not affect toxicity, whereas deleting a further three residues inactivated the polypeptide. Point mutations which individually converted arginine 48 and arginine 56 to alanine residues or which removed arginine 56 were also without effect on the catalytic activity of the toxin.

ABBREVIATIONS

APS	ammonium persulphate
ATP, dATP	adenosine triphosphate, deoxyadenosine triphosphate
b	base
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
Ci	curie
CTP, dCTP	cytidine triphosphate, deoxycytidine triphosphate
DATD	N N' - diallyltartardiamide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetra - acetate
g	gram
GTP, dGTP	guanosine triphosphate, deoxyguanosine triphosphate
kb	kilobase
l	litre
m	metre
min.	minute
ml	millilitre
ng	nanogram
RIP	ribosome-inactivating protein
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SV40	simian virus 40
TEMED	N,N,N',N' - tetramethyl - ethylenediamine
Tris	tris(hydroxyl)aminomethane
tRNA	transfer RNA

dTTP	deoxythymidine triphosphate
UTP	uridine triphosphate
v/v	volume per volume
w/v	weight per volume
ug	microgram
ul	microlitre

CHAPTER 1.

INTRODUCTION.

SECTION 1:1. GENERAL INTRODUCTION.

1:1:1. Historical background.

It has been known since ancient times that the seeds of Abrus precatorius L. (Leguminosae), and those of Ricinus communis L. (Euphorbiaceae), are highly toxic, but despite this, they have been used in a variety of medical treatments. Castor beans were used in classical Greek medicine and both plants are described in the Sanskrit work on medicine, *Susruta Ayuverda*, from the sixth century B.C. Extracts from both seeds have been used for a variety of criminal purposes. (Morton, 1977). Abrus seeds have found a wide variety of uses in their native South - East Asia and throughout the tropics. (Olsnes and Pihl, 1976). It was their widespread use as weights in this region that probably led to the spread of the plant throughout the rest of the tropics and subtropical regions along trade routes.

The Castor bean plant, (Ricinus communis), originated in Asia and Africa where its use in early medicine and its cultivation for oil were the probable causes for its spread to all parts of the world with a climate favourable to its growth. The castor bean was cultivated for its oil in ancient Egypt, the oil has since been used for a number of purposes. It is now used in medicine only as a laxative, but in earlier times, the oil, whole seeds and other parts of the plant were used against many diseases. The high toxicity of castor beans has been known since ancient times and at least 700 cases of intoxication in humans have been described. (Balint, 1974). In 1887, Stillmark gave the name " ricin " to the toxic component of the castor oil seed (Stillmark 1889a and b), whilst its extremely toxic and protein properties were first observed by Dixon, who also carried out the first

toxicological experiments with ricin on animals, though discussion as to whether it was a protein continued for many years.

The first extensive study of ricin was carried out by Stillmark identifying it as a protein with red blood cell agglutinating properties and being capable of precipitating serum proteins. He assumed that these effects could account for the toxic action of ricin on animals. Later, Hellin, in the same lab., discovered similar properties for abrin.

The assumption that cell agglutination was the reason for the toxic effect of abrin and ricin was soon challenged by the observation that the agglutinating properties of Abrus and Ricinus extracts could be separated from the toxic properties. Thus, when normal serum was added to the extracts, a precipitate was formed. The toxic property remained in the supernatant, whereas the agglutinating properties disappeared. Furthermore, the agglutinating ability of Ricinus extracts was more easily destroyed by treatment with pepsin - HCl than were the toxic properties.

Extensive immunological studies on abrin and ricin were carried out by Ehrlich, (1891, 1892). His work on abrin and ricin resulted in the discovery of some of the fundamental principles of immunology. He was able to show that immunisation of rabbits by feeding them small amounts of seeds or later by subcutaneous injections resulted in the formation of serum proteins capable of specifically precipitating and neutralising the toxins. Furthermore, a quantitative relationship was noted between the amount of antiserum and the amount of toxin it could neutralise and that anti-ricin did not protect against abrin and vice-versa, demonstrating that the phenomenon of immuno - specificity is associated with the antiserum. He could also demonstrate that, during pregnancy, immunity to the toxins was

transferred from the mother to the offspring by the blood and that after birth it may be transferred through the milk.

1:1:2. General characteristics of the plant toxins.

Since the first description of abrin and ricin many other toxic proteins of similar structure and activity have been isolated from a wide variety of plants, bacteria and fungi. (Olsnes and Pihl, 1982a and b, Barbieri and Stirpe, 1982, Jiminez and Vasquez, 1985, Stirpe and Barbieri, 1986,). It is thought that possibly all plants contain such proteins, all linked by common stuctural and functional properties.

The toxins abrin, ricin, (Olsnes and Pihl, 1976) and modeccin, (Olsnes et al., 1978a, Gasperi - Campani et al., 1978), are composed of two subunits: an A chain, of Mr = 30,000, and Pi between 4.8 and 8, which catalytically inactivates ribosomes, linked by a disulphide bond to a heavier B chain which binds sugars with the configuration of D - galactose. The B chain is classed as a lectin according to the definition of lectins suggested by Kocourek and Horejsi, (1981), although Goldstein and colleagues disputed this (Goldstein et al., 1980).

The demonstration that the B chains bind to neoplastic cells, giving to the toxins some anti - cancer activity (Lin et al., 1970a, b and 1971), prompted several researchers to look for new toxins which could have been useful for this purpose. It was this interest which led to the discovery of many of the toxins listed in table 1. (adapted from Jiminez and Vasquez, 1986, Barbieri and Stirpe, 1982, Stirpe and Barbieri, 1986.). These proteins were found in various parts of the plant (roots, leaves, seeds, sap), in concentrations varying from a

Table 1. The origins of plant ribosome inactivating proteins.

Plant	Family	Toxin	M.
Abrus precatorius	Leguminosae	abrin	65,000
Ricinus communis	Euphorbiaceae	ricin	62,057
Adenia volkensiae	Passifloraceae	volkensin	62,000
Viscum album	Loranthaceae	viscumin	60,000
Adenia digitata	Passifloraceae	modeccin	63,000
Phytolacca americana	Phytolaccaceae	pokeweed antiviral protein	29,000
Dianthus caryophyllum	Caryophyllaceae	dianthin	29,500
Gelonium multiflorum	Euphorbiaceae	gelonin	30,000
Agrostemma githago	Caryophyllaceae	agrostin	30,000
Hura crepitans	Euphorbiaceae	Hura crepitans inhibitor	28,000
Triticum sp.,	Graminae	tritin	30,000
Hordeum vulgare	Graminae	hordein	30,000
Momordica charantia	Cucurbitaceae	momordin	29,500
Saponaria officinalis	Caryophyllaceae	saporin	29,500
Luffa cylindrica	Cucurbitaceae	luffin	26,000
Asparagus officinalis	Liliaceae		32,500
Phytolacca dodecandra	Phytolaccaceae	dodecandrin	29,000

few micrograms to several hundred milligrams per 100g material. A variety of two subunit, (abrin, ricin, volkensin, viscumin, modeccin,), and one subunit toxins, (Pokeweed antiviral proteins, (PAP's), dianthins, gelonin, grain inhibitors), and four subunit toxins, (Abrus agglutinin, Ricinus agglutinin, viscumin lectin), were described.

The single chain proteins, like the active (A) chains of the toxins have the following properties:

1. in vitro. act by altering the 60S ribosomal subunit which could not then bind Elongation factor 2.

2. inactivate ribosomes in a less than equimolar ratio which suggested they acted as enzymes.

3. are moderately toxic to animals and mammalian cells, in this latter respect, their cyto-toxicity varies according to the target cell, macrophages seemed particularly susceptible.

4. are more toxic to virus - infected than to non - infected cells and could thus sometimes prevent virus replication in mammalian cells and certain plants.

5. have strong immuno-suppressive activity in mice.

6. can be rendered specifically cytotoxic by conjugation to molecules capable of binding to receptors on the cell surface (lectins, antibodies, sugars),

7. following insertion into liposomes, their toxicity can be much enhanced.

In the case of plant toxins containing 2 subunits, (eg. ricin), one of them, the B chain, is a glycosylated protein, the carbohydrate moiety of which always contains mannose and N - acetyl glucosamine. The B chain is involved, in all cases, in the interaction of the toxin with the sugar moiety of the cell receptor, whereas the A chain has an enzymatic nature displaying the same characteristics as the single

chain toxins above. The presence of the B chain facilitates the action of the A chain by binding to cell surfaces and playing some role in the internalisation of the A chain. Hence, the toxins having two subunits are more active in intact cells than toxins having a single chain which have difficulty entering the cell. The four subunit toxins (eg., Ricinus agglutinin), have essentially a structure similar to that indicated above for the two subunit toxins, but in the four subunit families, complexes of subunits of the same type are linked by weak interactions and therefore tetramers are formed, having two A chains and two B chains, (see section 1:2:1). The nomenclatures of the two and four subunit containing toxins are rather confusing because they are frequently referred to as lectins, having erythrocyte agglutinating activty to a certain extent. However, not all lectins inhibit protein synthesis. Furthermore, within the lectins of the same plant, cell agglutination does not appear to be related closely to cell toxicity. Thus ricin has weak cell agglutination activity but is a very toxic compound, whereas Ricinus agglutinin, poorer as a toxin, has stronger cell agglutination activity. Due to the general ambiguity of the term lectin, classification of these related proteins required a more specific nomenclature. To encompass all the various toxins sharing diverse sources yet similar functions and similar structures, the term Ribosome Inactivating Protein (RIP), was proposed, to define proteins ....." that inactivate eukaryotic ribosomes in a catalytic, (enzymic), manner, rendering the 60S ribosomal subunit incapable of binding the elongation factor 2, thus arresting protein synthesis." (Stirpe, 1982).

So far our knowledge is very limited as to why these plants produce toxins.

1.1.3. Bacterial and fungal toxins.

Ribosome inactivating proteins have so far been found exclusively in plants. The bacterial toxins from Corynebacterium diphtheriae, (Pappenheimer, 1977, 1982.),Pseudomonas aeruginosa. (Iglewski and Kabat, 1975,), and Shigella dysenteriae. (Olsnes et al., 1981, Reisbig et al., 1981) resemble toxic RIP's in their structure and inhibitory activity on protein synthesis. However, the mechanism of action of Diphtheria toxin and Pseudomonas toxin has been elucidated in detail and is different from the catalytic activity of RIP's. The best understood toxin is probably that produced by Corynebacterium diphtheriae (Diphtheria toxin). The gene for the toxin (the tox gene), is carried by a lysogenic bacteriophage present in certain strains of the host when grown in culture, the toxin is a non - essential protein both for the phage and the bacterium. However, the toxin clearly has survival value in the human population both for the phage and for the bacterium carrying it, (Pappenheimer, 1982). It is likely that the lesions induced in infected patients provide good growth conditions for the bacteria and when fragments of the membranes produced are coughed into the air, they represent efficient vehicles for the spread of the infection. It is less clear why Pseudomonas aeruginosa and Shigella dysenteriae carry toxin genes since it is not clear that the toxin provides the bacterium with a survival advantage. More recently, Shiga - like toxins have been found to be produced by various enteropathogenic E. coli strains, Vibrio cholerae. Salmonella typhimurium and Vibrio parahaemolyticus (Strockbine et al., 1985, Strockbine et al., 1986), and by E. coli isolates associated with hemolytic uremic syndrome (Karmali et al., 1983, Rose et al., 1985.).

Of the fungi, two species of Aspergillus, namely, Aspergillus giganteus and Aspergillus restrictus produce ribosome inactivating proteins, α - sarcin by the former (Olson and Goerner, 1965), and restrictocin and mitogillin by the latter. (Fernandez - Luna et al., 1985, Gavilanes et al., 1983, Lopez - Otin et al., 1984). All three proteins show considerable sequence homology, (Fernandez - Luna et al., 1985, Lopez - Otin et al., 1984, Conde et al., 1978), and all three cleave the 60S ribosomal subunit at the same site and thereby block the functional interaction of the EF - 1 - GTP aminoacyl - tRNA complex with the eukaryotic ribosome (Conde et al., 1978).

1:1:4. Evolutionary aspects.

The plant toxins appear to have developed by fusion of a gene for ribosome inactivating proteins with a lectin gene. Ribosome inactivating proteins that are structurally and functionally closely related to the plant toxin A chains (Ready et al., 1984, Xuejun and Jiahuai, 1986), are present in the majority of all plants which have been examined. Also lectins are common in plants (Brown and Hunt, 1978). Toxins of the A - B chain type are, however, found in only a few, not closely related plants. It is therefore likely that the different plant toxins have evolved separately by gene fusion. At least one of the plant toxins, ricin, is synthesised as one polypeptide chain (Lord, 1985). A 12 amino acid peptide linking the A and B chain is removed within the protein bodies (Harley and Lord, 1985). The castor bean genome apparently contains 6 ricin - like genes (Halling et al., 1985). Furthermore there is evidence to suggest that the B chain of ricin has evolved by gene duplication since it appears to be composed of two repeated units of similar sequence and

topology, each binding a galactoside. Each domain is seen to be composed of three versions of an ancient galactoside binding peptide in which the subdomains were free to undergo functional specialisation. (Lamb et al., 1985, Rutenber, et al., 1987). Why plants should have evolved such toxic proteins is not clear. However it is likely that the toxins play a role in the protection of the plants or their seedlings against animals. At least in one case, the protein is located in the cell wall matrix and it was speculated that cell destruction, eg. after insect bites, could release the A chain into the cytosol. Entry of the A chain like protein would stop cellular protein synthesis and multiplication and propogation of any viruses that entered the cells in connection with the mechanical damage (Ready et al., 1986). In fact antiviral properties of these proteins have been demonstrated in several systems (Ussery et al., 1977, Stevens et al., 1981, Stirpe, et al., 1981.).

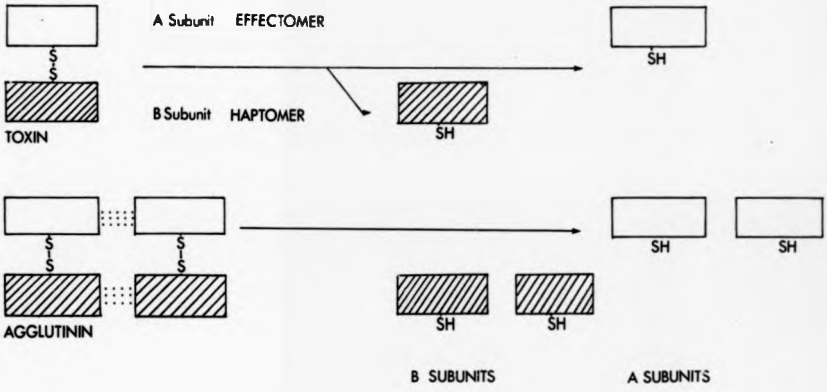
SECTION 1:2. THE STUCTURE OF PLANT RIP'S.

1:2:1 General structure.

Despite the fact that the toxins are produced by taxonomically unrelated plants, they show many striking similarities. Many of these can be attributed to great similarities in their structure.

Abrin, ricin and modeccin have molecular weights of 60,000 to 65,000 (Olsnes and Pihl, 1973a and 1973b; Nicolson et al., 1974; Olsnes et al., 1978a; Stirpe et al., 1981) and consist of two polypeptide chains joined by a single disulphide bond. (Olsnes and Pihl, 1972a and 1972b). The A chain or 'effectomer' is slightly shorter than the B chain or 'haptomer'. Abrus and Ricinus agglutinins consist of two heterodimers (see figure 1.2.1.) linked by weak interactions, although those holding the Abrus agglutinin subunits together have been found to be stronger than those holding the Ricinus agglutinin subunits together. (Saltvedt, 1976; Olsnes et al., 1974). Each heterodimer consists of two polypeptide chains (A' and B') again linked by a single disulphide bond.

Similarities in the primary stuctures of the toxins and agglutinins are considerable. Early studies by Gurtler and Horstman (1973) found that peptide maps of tryptic digests of the two Ricinus agglutinin chains were similar to those of the ricin chains. Nicolson et al., (1974), found similar patterns when they compared tryptic digest maps of whole ricin and whole Ricinus agglutinin. Confirmation of these similarities was provided by the determination of the primary amino acid sequence of ricin by Funatsu and colleagues (Nanno et al., 1975; Funatsu et al., 1978; Funatsu et al., 1979). The carbohydrate


A Subunit EFFECTOMER
B Subunit HAPTOMER
TOXIN
SH
SH
AGGLUTININ
SH
SH
SH
SH
B SUBUNITS
A SUBUNITS

composition of both ricin chains was also determined by these authors. More recently, Butterworth and Lord (1983) showed that both chains of ricin are encoded by a single mRNA and are not the products of two distinct transcripts. During ricin biosynthesis in Ricinus endosperm cells, an N - terminal signal sequence directs its synthesis to the rough endoplasmic reticulum. After deposition in the lumen of the ER, proricin is transported via the Golgi and Golgi derived vesicles to the protein bodies where an acid endopeptidase cleaves the precursor to liberate the A and B chains. (Harley and Lord, 1985; Lord, 1985; Lord, 1985a). It is assumed that the other heterodimeric cytotoxic lectins are likewise synthesised via a single precursor polypeptide. A cDNA sequence for ricin (Lamb et al., 1985), and Ricinus communis agglutinin (Roberts et al., 1985) were subsequently described, followed shortly by a genomic sequence (Halling et al., 1985) which confirmed the proenzyme structure of ricin and also revealed that the gene has no introns.

1.2.2. The 3-D structure of ricin determined by X - ray crystallography.

The X - ray crystallographic structure of ricin has recently been determined at 2.8A resolution. (Montfort et al., 1987) Prior to this the only ribosome inactivating protein of which the 3D structure was known was trichosanthin, a single chain toxin derived from the plant Trichosanthes kirilowii (Pan et al., 1982) and even then this was at too low a resolution to determine any fine structural detail.

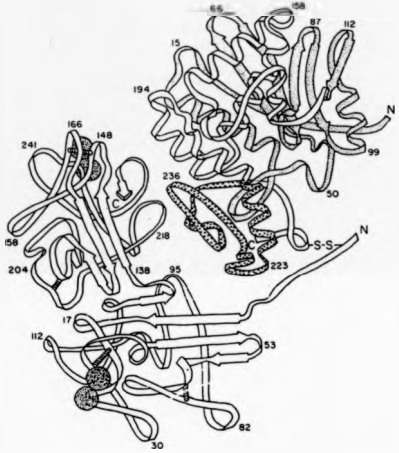


FIGURE 1.2.2 THE 3D STRUCTURE OF RICIN

Taken from Montfort et al., 1987.

General features of the B chain.

Figure 1.2.2. shows a ribbon representation of the ricin backbone adapted from Montfort et al., 1987. The B chain occupies the lower left portion of the figure. It is roughly 70Å long, about 30Å wide and is composed of two separate folding domains. Each domain is a roughly 30 - Å sphere and binds one lactose dimaccharide, as predicted (Villafranca and Robertus, 1981).

General features of the A chain.

The A chain is a more globular protein, situated in the upper right of figure 1.2.2. The protein has the shape of an oblong disc, approximately 55Å long, 45Å wide and only about 35Å thick. The carboxyl 57 residues fold into a protruding domain that interacts with the B chain by sliding into the cleft between the two B chain domains. A disulphide bond is formed between residues 259 of the A chain and 4 of the B chain. The amino 8 - 10 residues of the B chain form a long arm which interacts with the carboxyl terminal domain of the A chain. As a result, the inter-chain disulphide bond is in an exposed region quite far removed from the main body of the B chain.

Specific features; structure - function relationships.

The A chain folds into three domains and exhibits a substantial amount of secondary structure. The amino - terminal 117 residues comprise the first domain which is dominated by a five stranded beta - pleated sheet. The second domain is composed of residues 118 - 210 and is dominated by five alpha helices. The third domain, residues 211 - 267 forms a compact, disc - like domain, roughly 25 - 30Å across and 15 - 20Å thick. This domain interacts with the first two domains of the A chain and is also involved in a strong interaction with the B

chain. The protein has a reasonably prominent cleft created at the interface between all three domains, it is very likely that this cleft represents the active site of the ricin A chain subunit. Whilst the A chain is in association with the B chain this would be more aptly termed a latent active site since a conformational rearrangement may take place upon release of the A chain. This is suggested by the fact that the cleft has good access to solvent in the heterodimer yet the heterodimer itself is inactive.

The B chain is a lectin with an affinity for galactosides. The protein has been known for some time to bind two galactosides in a non - cooperative fashion (Zentz et al., 1978; Houston and Dooley, 1982). As discussed in section 1:4:3., the B chain is a gene duplication product, showing about 32% amino acid identity between the two halves. As can be seen in figure 1:2:2., the two domains have identical topologies, the lactose binding site of each domain is shown as a pair of circles. Work is currently underway in this lab to determine the contribution of specific amino acid residues to the galactose-binding function of ricin B chain by site-directed mutagenesis of the cloned gene followed by expression and assay (Richardson et al., 1988). It is interesting to note that the two domains of the B chain are the product of a gene duplication, have similar folding topologies, and bind galactose in similar ways; yet, the residues involved in that binding site are not directly related by the gene duplication. The implication of the two apparently non - homologous binding sites in two clearly homologous domains for the evolution and function of the B chain will be addressed in section 1:4:3.

It is known that the A chain of ricin is not enzymically active, unless it is released from the B chain (Olsnes and Pihl, 1982a); it is also known that the release exposes a nonpolar area on the A chain

the A chain which may aid in membrane transport. (Houston, 1982). It is quite likely that release from the B chain causes a conformational change in the A chain which activates the enzyme function and also alters the nonpolar surface to facilitate membrane binding. Data obtained from recently crystallised A chain expressed from a gene cloned in *E. coli* may shed light on this putative conformational change. (Robertus et al., 1986).

SECTION 1:3 TOXIC EFFECTS

1:3:1. Effects in animals and man.

By far the best studied of the toxins are abrin, ricin (Fodstad et al., 1976, 1979; Olsnes and Pihl, 1976) and modeccin (Gasperi - Campani et al., 1978; Sperti et al., 1979). Different animal species exhibit different sensitivities to the toxins. For instance, calculated on a weight basis, the guinea pig is more sensitive to ricin than the mouse, (Ehrlich, 1891, 1892), 1g being sufficient to kill 1.5 million guinea pigs. Meissner and co - workers have published a comparison of the sensitivity of different animals to castor - oil seed among the higher animals, the domestic hen is the most resistant, whilst the horse is the most sensitive (see Barbieri and Stirpe, 1982). The resistance of the frog is also notable, at 4°c, frogs injected with 400ug of ricin survive for more than two weeks. Indeed, the body temperature seems to be of importance in determining the susceptibility of the organism. The pathological effects and ultrastructural lesions caused by RIP intoxication are well-documented. (Olsnes and Pihl, 1976, Rauber and Heard, 1985). The toxins are lethal to most cells studied but it is not clear which organs are the primary targets to acute intoxication. On the basis of experiments with protective antibodies, (ie, anti-ricin), it was proposed that damage to the central nervous system is the major effect of ricin (Foxwell et al., 1985) although Fodstad found no evidence for this. Upon injection of ricin, there is a latent period observed of some hours before the symptoms of intoxication develop and the animal dies after 10 hours or more. Even when very high amounts of toxin,

(several mg), are injected into mice, they do not die earlier than after about 10 hours. (Fodstad et al., 1976).

After injection of high doses of toxin, the most common findings are haemorrhages in the intestine, mesenterium and omentum. Before death, the body temperature decreases and the animals shiver in a characteristic way. Injection of smaller and sublethal doses of ricin and abrin cause smaller haemorrhages and multiple necroses in the liver and kidneys. If the ricin is tagged by radioactive labelling and injected into mice, the highest concentration is found in the liver and spleen. (Olsnes and Pihl, 1976).

In humans, the most common form of intoxication is ingestion of seeds or by inhalation of dust. One or a few Abrus or Ricinus seeds may, when chewed and swallowed, be lethal. The most common symptoms are nausea, vomiting, pain in the stomach, cramps, colic, diarrhoea, haemorrhage from the anus, anuria, dilation of the pupillae, fever, thirst, burning in the throat, headache and shock symptoms. Death occurs from exhaustion or cramp. The most common autopsy findings are multiple ulcera in the stomach and small intestine with numerous haemorrhages.

Abrin, ricin and modeccin can be detected in biological material by preparing extracts from the material and testing them on cells in culture in the presence and absence of antibodies. In this way, quantities down to about 1ng of toxin can be detected. The value of this test, however, would depend on the size of the dose given and on how soon after intoxication the sample was taken, since the toxins are degraded with a half - life of 5 hours (ricin), and 17 hours, (abrin) (Olsnes and Pihl, 1976).

Single chain RIP's presumably act in the same way in vivo as they do in vitro (Montanaro et al., 1978). The differences in

toxicity noted between these and the two - chain toxins could be accounted for by the lack of carbohydrate binding subunit which is necessary for the entry of the A chain into the cell.

1.3.2. Protein synthesis inhibition in cultured cells.

The first demonstration that the effect of abrin and ricin on cells in culture is inhibition of protein synthesis was by Lin et al., (1970b, 1971). Somewhat later, the DNA synthesis decreases and still later also, the RNA synthesis is inhibited (Refsnes et al., 1977; Nicolson et al., 1973; Refsnes et al., 1974). These results were confirmed elsewhere with various animal and human cell lines. The data indicated that the toxic effect is primarily due to inhibition of protein synthesis. (Olsnes et al., 1974). After addition of abrin and ricin to the cell culture medium, a lag period occurs before inhibition of protein synthesis is apparent in the cells (Olsnes and Pihl, 1976), the lag is decreased by increasing toxin concentration but even at high concentration cannot be reduced below a minimum of about 20 - 30 min. (Refsnes et al., 1974; Olsnes et al., 1976). This is presumably the time it takes for sufficient of the A chain to be internalised for the intoxication to be measured. In this lab, intoxication is measured by the depletion of ^{35}S methionine incorporation into labelled protein. Intoxicated cells undergo early surface changes becoming more irregular although it is observed that the cells continue to exclude trypan blue for several hours after protein synthesis has ceased (Nicolson et al., 1974; Nicolson et al., 1975), indicating the surface membranes remain intact for a long period of time. Eiklid and co - workers demonstrated that one molecule of ricin A chain is enough to kill a cell (Eiklid et al., 1980

)provided that it reaches the cytoplasm after having crossed the plasma membrane. If their findings are extended, the percentage of protein synthesis inhibition is a very close estimate of the percentage of cell death. Different cell lines show differing sensitivities to the RIP's. The most sensitive appear to be peritoneal macrophages, human lymphocytes and to a lesser extent, virus-infected and virus - transformed cells. (Barbieri and Stirpe, 1982). The variable sensitivity of cell lines from different sources may in part be a reason for the variation noted in the sensitivity of different animals to intoxication.

SECTION 1:4. FUNCTIONS OF THE A AND B CHAINS.

1:4:1. Different biological properties of the two constituent polypeptide chains

Jacoby, in 1902, observed that the agglutinating properties of Abrus and Ricinus extracts could be separated from the toxic properties and that the agglutinating ability of Ricinus extracts was more easily destroyed by treatment with Pepsin - HCl than were the toxic properties.

In 1972, Olsnes and Pihl showed that when abrin and ricin were treated with 2 - mercaptoethanol, to reduce the interchain disulphide bond, the toxins lost most of their ability to inhibit protein synthesis in intact cells and to intoxicate animals (Olsnes and Pihl, 1972b). If the 2 - mercaptoethanol was then removed by dialysis, the interchain disulphide bond was reformed and the toxicity was fully restored (Olsnes et al., 1974; Funatsu et al., 1973a). It was also

shown that abrin and ricin inhibited protein synthesis in a rabbit reticulocyte lysate and that the reduced toxins were much more potent inhibitors than the intact toxins. (Olsnes and Pihl 1973a). Furthermore, when the A and B chains were tested separately, the A chain strongly inhibited protein synthesis in the cell - free system whereas the B chain lacked this ability. Since only the intact toxin has a toxic effect on animals and intact cells, it was clear that the B chain must have some important function. The first indication that the B chain binds to cell surfaces, whereas the A chain does not came from evidence obtained in haemagglutination experiments. Human erythrocytes pretreated with intact ricin or with B chain were agglutinated by an antiserum directed specifically against ricin whereas erythrocytes pretreated with A chain were not agglutinated under the same conditions. Only the B chain bound to a Sepharose column, this binding could be abolished by the addition of galactose. Similarly, B chain covalently bound to a Sepharose column could bind erythrocytes, whereas A chain covalently bound to a similar column could not. (Olsnes et al., 1974; Olsnes and Pihl, 1973a). The results indicated that the toxic action of ricin is associated with the A chain and that the B chain functions as a carrier moiety which binds the toxin to the cell surface, a binding which is reversible and involves galactose containing receptors. Experiments with radioactive B chain and whole ricin have supported this by showing that the ability of the toxin to bind to the cell surface is associated with the B chain and that unlike the A chain, the B chain has the same biological activity whether free or present in the intact toxin (Sandvig et al., 1975). Furthermore, when purified A and B chains were applied to a Sepharose 4B column, only the B chain was retained and could later be eluted with galactose,

whereas the A chain passed through (Olsnes et al., 1974; Olsnes and Pihl, 1973a).

Olsnes and colleagues (1974a), showed that, when mixtures of abrin A chain / ricin B chain and *vice - versa* are dialysed, the two chains combine to form hybrid molecules. The hybrids were as toxic to living animals and cells in culture as are native abrin and ricin. Furthermore, although in culture, antibodies raised against each of the four constituent polypeptides show no cross reactivity, both anti-abrin and anti-ricin protect animals and cells against the toxic effect of the hybrid molecules. Futhermore, antibodies raised against either of the two peptide chains of the hybrid molecules are equally effective in protecting against the toxic effect.

1:4:2 Function of the A chain.

1:4:2:1 Enzymic inactivation of the 60S ribosomal subunit

The toxin A chains inhibit protein synthesis by inactivating the ribosomes (Olsnes and Pihl, 1972b; Montanaro et al., 1973). This was convincingly demonstrated in experiments where ribosomes and supernatants were treated separately with the toxin A chains (Olsnes et al., 1973; Benson et al., 1975). After the treatment, specific anti-toxins were added to prevent further activity of the A chains and cell - free systems were reconstituted from toxin-treated ribosomes and untreated supernatant factors and *vice-versa*. Only when the ribosomes had been pretreated was protein synthesis in the cell - free system inhibited. Separate treatment of the 40S and the 60S ribosomal subunits with the toxin A chains revealed that abrin and ricin specifically inactivated the 60S subunit whereas they had no effect on the 40S subunit, in accordance with the findings of Sperti

and colleagues (1973). It was also shown that bacterial (Olsnes et al., 1973; Greco et al., 1974) and mitochondrial ribosomes were resistant (Greco et al., 1974). Whether toxin A chains inactivate plant ribosomes has never been clearly established and few studies have been made. Nevertheless, there is some evidence that RIP's do not affect ribosomes obtained from homologous plants. Pokeweed anti-viral protein, (PAP) and tritin, respectively, did not inactivate ribosomes from pokeweed (Owens et al., 1973) and from wheatgerm (Coleman and Roberts, 1981). On the other hand, PAP inhibited protein synthesis by cowpea and wheatgerm ribosomes (Owens et al., 1973), and ricin those of wheatgerm (Cawley et al., 1977, 1979; Lugnier and Rether, 1981), although at concentrations much higher than those effective on mammalian ribosomes. This suggested that the wider the evolutionary gap between the source of RIP and of its target ribosomes, the greater the inactivation is likely to be. This led to the suggestion that

1, ribosomes differ between plants, possibly with species specificity,

2, many, if not all, plants possess proteins capable of recognising ribosomes different from their own and of inactivating them. (Stirpe, 1982).

The question of plant ribosome inactivation by toxin A chains is currently under investigation in this lab.

Early experiments by Olsnes and colleagues showed that one toxin molecule inactivates a large number of ribosomes (Olsnes and Pihl, 1972a, 1972b, 1972c; Olsnes et al., 1973), indicating that the toxins act by a catalytic mechanism. Subsequently the kinetics of ribosome inactivation by ricin A chain was demonstrated (Olsnes et al., 1975). Highly purified toxin A chains inactivate salt washed ribosomes in simple buffer solutions at a rate of about 1500 ribosomes per minute per toxin A chain. The Q_{10} was about 1.8 and the K_m about

1 - 2 x 10^{-7}M. Furthermore the inactivation could be stopped at any time by adding the specific anti - A chain antibodies (Olsnes et al., 1975), the observation that the toxins act catalytically was made by several other authors (Montanaro et al., 1973; Carrasco et al., 1975). This clearly demonstrated that the A chains are acting directly on the 60S ribosomal subunits.

Similarly, in intact cells, the 60S ribosomal subunit has been shown to be the site of action of the toxins. Onozaki and colleagues (1975), showed that, when ribosomes were isolated from toxin - treated cells and their ribosomal subunits separated and tested together with the complimentary subunits from untreated cells, the 60S subunits from the toxin - treated cells were inactive whereas the 40S subunits remained active. Also the supernatant factors remained active in the toxin - treated cells.

1:4:2:2 **Steps inhibited in protein synthesis.**

The observation that the polysome structure was preserved in the presence of the toxin A chains indicated that the toxins inhibited some step involved in peptide chain elongation (Olsnes and Pihl, 1972a and 1972c). This was further supported by experiments showing that the toxins prevent the disappearance of polysomes in the presence of aurin tricarboxylic acid which selectively inhibits reinitiation of new peptide chains. Further studies showed subsequently that the toxins do not inhibit the binding of puromycin to growing peptide chains, indicating that the toxins do not inhibit the formation of the peptide bond (Montanaro et al.,1973; Benson et al., 1975; Carrasco et al., 1975).

Carrasco and colleagues (1975) reported that ricin inhibits the enzymatic binding of aminoacyl - tRNA to ribosomes, whereas

Montanaro and colleagues (1973), could not demonstrate this effect. The toxin inactivated ribosomes exhibit a reduced ability to hydrolyse GTP in the presence of the elongation factors EF - 1 (Benson et al.,1975) and EF - 2 (Montanaro et al., 1973; Benson et al., 1975; Sperti et al., 1975a). Furthermore, experiments with ^{14}C labelled adenosyl - diphosphoribose - EF2 as well as ^{3}H - EF2 have shown that the binding of EF2 in the presence of GTP or the analogue GMPCP, gave the same results (Carrasco et al., 1975). By pretreating ribosomes with EF2, Fernandez - Puentes and colleagues were able to demonstrate that prebound EF2 protected the ribosome against inactivation by toxin A chains. These data indicated that the toxins act on the ribosomes at a site identical with or close to the binding site for EF2 and changes this site in such a way that the affinity for EF2 is reduced. High concentrations of EF2 can partly overcome the protein synthesis inhibition by the toxin A chains (Olsnes et al., 1975), showing that the affinity is only reduced and not completely abolished.

1:4:2:3. Liberation of the A chain from the B chain.

Abrin and ricin treated with 2 - mercaptoethanol have a greatly increased ability to inhibit cell - free protein synthesis. This increase was shown not to be due to a requirement of free SH groups since the inhibitory effect of reduced toxins or the isolated A chains was only moderately lowered after treatment with N - ethylmaleimide which binds irreversibly to free SH groups (Olsnes et al., 1975). Lewis and Youle recently confirmed this observation in showing that the disulphide bond plays no role in toxicity other than to hold the two subunits together at low concentrations. (Lewis and Youle, 1986). When intact toxins were added to cell free systems, the rate of protein synthesis inhibition was first low and then increased

rapidly with time (Olsnes et al., 1976; Olsnes and Pihl, 1972a and 1972c). The kinetics were consistent with the view that the A chain must be liberated before protein synthesis inhibition occurs. If reducing agents are not present in the system, even high concentrations of intact toxin will not inactivate ribosomes. In rabbit reticulocyte lysate, and probably also in the cytoplasm of other cells, enough reducing agents are present to split the interchain S - S bond in abrin and ricin rapidly. The speculation that glutathione accounted for this reducing activity was confirmed by Lewis and Youle, (1986) by using diethyl malonate to reduce glutathione levels - this reduced the toxicity of intact ricin but not that of reduced ricin. It is not clear whether the disulphide bond plays a role in toxin entry into cells eg. by disulphide exchange with free SH groups in membrane molecules, or if it only prevents dissociation of the A and B chains at low toxin concentrations.

1:4:2:4 The mechanism of action of diphtheria toxin and *Pseudomonas* exotoxin A.

Diphtheria toxin

Diphtheria toxin (DT) is synthesised by *Corynebacterium diphtheriae* as a single polypeptide chain (Mr 62000). The toxin contains an arginine - rich region which is readily cleaved by trypsin and other proteolytic enzymes of similar specificity. This yields a 'nicked' toxin consisting of two subunits, A (Mr 22000) and B (Mr 40000), linked by a single disulphide bond (Gill and Dinius, 1971; Collier, 1975). The A chain catalyses the ADP - ribosylation of an unusual amino acid (called diphthamide) in elongation factor 2 (EF-2). (Van Ness et al., 1980). The modified EF 2 is unable to exchange GDP with GTP (Burns et al., 1986). EF 2 is required for the

translocation of peptidyl - tRNA from the A site back to the P site on the ribosome after the peptide bond has been formed and so protein synthesis stops when EF 2 is inactivated. It is thought that a single or a very few molecules of A chain can inactivate enough EF2 to kill the cell (Yamaizumi et al., 1978).

Pseudomonas aeruginosa exotoxin A

Like diphtheria toxin, Pseudomonas exotoxin A (PA), inhibits protein synthesis in eukaryotic cells by catalysing the transfer of the ADP ribosyl moiety of oxidised NAD onto EF 2. Biochemical studies have shown that PA is similar to DT in a number of other ways;

1. it is of similar size (Leppla, 1976),

2. it is secreted as a single polypeptide chain containing disulphide bridges and no free SH groups (Lory and Collier, 1980),

3. it is a proenzyme that must undergo alterations in covalent structure before ADP - ribosyltransferase activity is expressed (Lory and Collier, 1980; Vasil et al., 1977),

4. it appears to modify the same site on EF 2 and possess a similar binding constant for NAD^+,

5. it is produced maximally in iron deficient medium (Bjorn et al., 1978),

The enzymic and binding functions are associated with different domains of the protein. X - Ray crystallography of PA shows that it consists of three domains (Allured et al., 1986). The function of each of the three domains was identified by deletion of the gene expressed in E. coli (Hwang et al., 1987), domain three being responsible for the ADP - ribosylation activity.

1.4.3. **Function of the B chain.**

The B chain binds the toxin to the cell surface during the course of intoxication. Additional functions required for penetration into the cytosol may be associated with particular domains in either the A or the B chain. Thus, hydrophobic regions in diphtheria toxin fragment B are involved in the penetration of fragment A (Boquet *et al.*, 1976; Lambotte *et al.*, 1980; Sandvig and Olsnes, 1981), and a hydrophobic region in ricin A chain may be of imortance for entry of the A chain into the cytosol. Also in addition to its role in binding, the ricin B chain may play a role in the entry of the A chain. The fact that removal of carbohydrates from ricin B chain, but not from ricin A chain reduces the toxic activity of the molecule could be relevant in this connection. (Utsumi *et al.*, 1984).

The B chain is known to bind two galactosides in a non - cooperative manner. (Zentz *et al.*, 1978; Houston and Dooley, 1982). In one study, the dissociation constants of the two sites were found to be 0.03mM and 0.4mM (Zentz *et al.*, 1978), whilst in another study using fluorescent galactose analogues showed more nearly equal affinities for the two sites (Houston and Dooley, 1982). Recent chemical evidence has implicated Tyrosine 248 in the 'strong' galactose binding site (Nims *et al.*, 1986), and an undetermined tryptophan in the 'weak' site. (Hatakeyma *et al.*, 1986). All of the two chain toxins have lectin properties in that they bind to carbohydrates at the cell surface and, once bound, they can be released by addition of competing sugars. (Olsnes and Pihl, 1976, Olsnes and Sandvig, 1983). Although lectins with specificity for a large number of different sugars have been described (Brown and Hunt, 1978), the plant toxins all have affinity for galactose. It is unlikely that this is a coincidence, since

among the non - toxic lectins, specificity for galactose is not more frequent than specificity for eg. mannose or N - acetyl - glucosamine. Although galactose and lactose compete for binding to cells of all five plant toxins, there are clearly differences in their binding specificity. Thus, while abrin and ricin bind to 3 x 10^7 sites per HeLa cell, modeccin binds only to 2 x 10^6 sites on HeLa cells and BHK cells (Sandvig et al., 1976; Olsnes et al., 1978a, Gleeson and Hughes, 1985). Also, galactose and lactose inhibit to different extents the binding of the different toxins to cells.

Many surface carbohydrates have terminal sialic acid residues with galactose as the penultimate sugar. Treatment of cells with neuraminidase therefore exposes previously hidden galactose residues. In accordance with this, neuraminidase treatment also increases the ability of cells to bind the plant toxins and as a consequence, the toxin sensitivity of the cells (Rosen and Hughes, 1977; Sandvig et al., 1978).

A large number of different surface glycoproteins and glycolipids contain galactose. It is not clear if all of these molecules can serve as functional binding sites for the toxins in the sense that they promote transfer of the toxin to the cytosol. It is conceivable that only a subpopulation of the toxin binding sites is able to facilitate penetration of the toxin into the cytosol.

The plant toxins contain complex carbohydrate chains and may also bind to cells through these oligosaccharides, since animal cells have receptors for carbohydrates. It was shown that mannose receptors present on reticuloendothelial cells are efficient in internalising ricin in the presence of lactose (Youle et al., 1981). In fact, even free A chain which contains one oligosaccharide chain is toxic to such cells (Simmons et al., 1986). Modification of the carbohydrates on

ricin reduced the uptake by the mannose receptor pathway. (Thorpe et al., 1985a; Skillater et al., 1985; Skillater and Foxwell, 1986).

Evolution of the B chain.

The ricin B chain is divided into two homologous domains consisting of residues 151 - 183 and 187 - 226(Villafranca and Robertus, 1981), folded identically (Montfort et al., 1987) and of similar structure, each built from three serial repetitions of a primitive unit.(Rutenber et al., 1987). Interestingly, these peptides are homologous to residues 168 - 225 of Discoidin 1, a galactose binding protein isolated from the slime mould Dictyostelium discoideum. That observation supported the contention that the 40 residue disulphide containing peptide was an ancient galactose binding unit, coded by an exon - sized piece of DNA, which has been incorporated into a variety of proteins. Since this unit is found both in higher plants and slime moulds, it is clear that the putative galactose binding unit must have already existed prior to the divergence of the plant and animal kingdoms - perhaps 1 billion years ago (Dayhoff, 1978).

It was proposed that the initial 40 residue peptide had a loop which bound galactosides in a shallow pocket formed partially by an aromatic residue, further stabilised by hydrogen bonding to an Asn residue. Such a primordial peptide may not have bound sugars with great affinity and probably lacked the structural stability of a complete protein. Nevertheless, if it sequestered sugars at even a low level, it could have conferred a selective advantage on its host. It seems likely that the primitive unit trimerized through interactions of Trp and Ile residues (Rutenber et al., 1987). Presumably this trimer bound galactose at each of the three equivalent recognition sites.

Recently, the trimer duplicated to form the modern two - domain B chain, in this two domain structure, access to one of the galactose binding sites became sterically hindered by the presence of the second domain.

It had been shown that the A chain of ricin has an evolutionary history independent of the B chain (Ready et al., 1984) and that ricin is the product of the fusion of the two separate genes (Lamb et al., 1985; Halling et al., 1985). This fusion must have occurred after the gene for B chain had undergone its most recent duplication to modern form. That is, we see A chain - like ribosome inactivating proteins existing either independently of any B chain or in the presence of a complete B chain, however, no A chain like protein has been discovered associated with a half B chain, that is, with a single galactose binding domain. This gene fusion seems to be part of a chain of events which converted the A chain from a passive defence role, as suggested for PAP (Ready et al., 1986), to an active role in which the toxin could penetrate foreign cells. It is interesting to note that another plant lectin, wheatgerm agglutinin, has evolved by repeated duplications and fusions of a gene coding for a small ancestral folding unit (Wright et al., 1985). The case for multiplication of a primitive unit to form B chain also seems clear from the internally homologous amino acid sequence patterns and structural features seen within the units.

A peptide strikingly similar in sequence to ricin B chain half sub - domain, and presumably monofunctional, can be seen in the primary structure of the galactose binding protein from E. coli (Mahoney et al., 1981).The proposal made by Rutenber and colleagues is that this simple monofunctional loop structure is a discrete element in protein evolution, and an extremely ancient one indeed. The idea of

a simple short loop structure, capable of binding a biologically important ligand and acting as a kind of primordial building block, is appealing. A key aspect of the ricin B chain structure is that it demonstrates very clearly and simply features of significance fror the evolution of genes and proteins, particularly in the selective advantage gained in fusing genes for protein building blocks. Fusion can give increased stability to aggregates, but more importantly, opens up new evolutionary pathways for the proteins themselves.

1:4:4. **Endocytosis and intracellular routing.**

It is well established that ricin labelled with horse radish peroxidase or with colloidal gold is bound to the cell surface and that the conjugates are then slowly taken up by endocytosis (Gonatas *et al.*, 1980; van Deurs *et al.*, 1985). This occurs partly by the coated pit/coated vesicle pathway, but also by a mechanism not involving coated vesicles. Thus, when the coated pit/coated vesicle pathway was blocked by a method involving hypotonic shock and subsequent K^+ - depletion (Moya *et al.*, 1985), orby acidification of the cytosol (Sandvig *et al.*, 1987), the uptake of ricin was only slightly inhibited. Since also the uptake of the fluid phase marker, lucifer yellow, was not markedly reduced (Sandvig *et al.*, 1987), the data indicate that ricin is taken up by an alternative pathway of endocytosis.

In macrophages ricin is taken up both via the galactose containing cell surface stuctures and via the mannose receptor (Simmons *et al.*, 1986). In fact the latter mechanism appears to be the most efficient one. While NH_4Cl sensitises cells to ricin taken up by the normal route, it was found to protect against toxin taken up by the mannose receptor. (Simmons *et al.*, 1986).

Ricin is first delivered to the endosomes and it then appears in the Golgi complex (van Deurs et al., 1986).

The trans - Golgi reticulum is involved in packaging of proteins for export (Griffiths and Simons, 1986). It is in accordance with the observation that endocytosed ricin appears to accumulate in this organelle, that a considerable part of the internalised ricin is recycled back to the cell surface by a process that is inhibited at temperatures below 20°c (Sandvig and Olsnes, 1979). The finding that part of the endocytosed ricin is exocytosed rapidly, whilst the other part is recycled much more slowly is in accordance with the theory that the recycling occurs by two different routes. (Sandvig and Olsnes, 1979).

It is thought that modeccin is internalised by a process dissimilar to the one proposed for ricin, abrin, viscumin and shigella toxin above, and more akin to the well described process by which diphtheria toxin and Pseudomonas exotoxin A are internalised (Olsnes and Sandvig, 1987).

Transport to the cytosol.

Unlike diphtheria toxin, Pseudomonas exotoxin or modeccin, which require a low pH for the transfer of the A chain to the cytosol (Sandvig and Olsnes, 1987), ricin, abrin and viscumin do not. In fact,, these toxins enter most efficiently when the pH is slightly alkaline; amines and other compounds that increase the pH in intracellular vesicles increase the sensitivity of cells to the toxins (Sandvig et al., 1979; Nakada et al., 1981).

Ricin has no exposed hydrophobic domains at any pH value, unlike diphtheria toxin, however, upon separation of the two polypeptide chains, they both exhibit hydrophobic and interacting domains

(Montfort et al., 1987), both of which may cooperate in the entry of the A chain into the cytosol.

The entry of abrin, ricin, modeccin, shigella toxin, viscumin and Pseudomonas exotoxin A requires the presence of Ca^{++}, and a pH higher than 6.0 (Sandvig and Brown, 1987). Wellner and colleagues (Wellner et al., 1984) described a mutant chinese hamster ovary cell line resistant to ricin and Pseudomonas exotoxin A. The resistance, which behaved as a dominant character, was associated with incorporation of palmitic acid into two cellular proteins with molecular weight of 30,000. Possibly these proteins play a role in endocytosis or intracellular routing.

SECTION 1:5. **RECENT DEVELOPMENTS.**

Progress in understanding the mechanism of action of bacterial and fungal toxins was well ahead of that concerning the plant ribosome inactivating proteins. The use of ricin has been prototypic in the design and construction of immunotoxins (see section 1:6), yet herein lay a paradox. Despite its widespread use in arming monoclonal antibodies for targeted cell killing (immunotoxins), the exact mechanism of its catalytic activity was not understood. Therefore, the cDNA cloning (Lamb et al., 1985) and later, the genomic cloning (Halling et al., 1985), of preproricin represented a fundamental breakthrough in the search for elucidating the mechanism of ricin A chain activity. With the range of DNA manipulation techniques and the X - Ray structure of ricin (Montfort et al., 1987) available progress was envisaged in determining structure - function relationships by delineating the regions of the gene which determine specific functions. Knowledge of the portions of the ricin A chain gene important in determining its function would be of great value for the construction of recombinant immunotoxins. (see section 1:6:6).

1:5:1. Comparison of the DNA sequences encoding ribosome inactivating proteins.

It was some time after the publication of the ricin cDNA and genomic sequences that the cloning of other ribosome inactivating proteins was reported. To date, few sequences have been published, although attempts to clone other RIP's are currently in progress. The cloning of cDNA's encoding trichosanthin (Xuejun and Jiahuai, 1986), Shiga - like toxin from E. coli phage H19B (Calderwood et al., 1987; De Grandis et al., 1987), Pseudomonas exotoxin A (Gray et al., 1984),

diphtheria toxin (Greenfield et al., 1983; Leong et al., 1984) has been achieved. Recently, the amino acid sequence of abrin was determined (Funatsu et al., 1988). By a detailed comparison and alignment of RIP genes, predictions can now be made as to the nature of the nature of the sequences encoding the active site of the RIP's. The assumption being made here, is that homologous sequences represent homologous functions; the function shared by all of these proteins being ribosome inactivation, presumably by interfering in some unknown way, with the elongation process of translation on eukaryotic ribosomes. By then aligning amino acids conserved between a significant proportion of the genes with the active site cleft on the 3 - D structure of ricin A chain proposed by Montfort and colleagues (1986) some degree of certainty in predicting that the amino acids are involved in the catalytic activity of the RIP is ensured. By use of the wide variety of DNA manipulation techniques available it is expected that expression of the modified gene and assay would reveal the nature of the active site.

The first such sequence comparison involved that of ricin A chain and trichosanthin (Xuejun and Jiahuai, 1986) and revealed a striking degree of homology. The identical and conserved residues comprise at least 56% of the trichosanthin sequence. It is believed that the sequence similarity between trichosanthin and ricin A chain is by no means a random event. Probability estimation of the alignment made reinforced this. It is reportedly very rare for an identical pentapeptide to appear in different proteins (Kabsh and Sander, 1984) the probability is estimated to be about 1×10^{-6}. One may then expect the frequency of the appearance of any identical pentapeptide in both trichosanthin and ricin A chain to be about 0.05. In fact, three identical polypeptides have been found. One is GYRAG (according to

the one - figure notation of amino acids, (Biochem J. 113, 1 - 4)), in residues 80 - 84 of trichosanthin and 83 - 87 of ricin A chain. The second is LRENI in residues 128 - 132 of trichosanthin and 133 - 137 of ricin A chain. The third is SEAAR in residues 166 - 170 of trichosanthin and 176 - 180 of ricin A chain. Thus the remarkable sequence homology between these two proteins seems not to be coincidental and may well be of biological significance.

Funatsu and colleagues (Watanabe and Funatsu, 1986) chemically modified arginine residues in ricin A chain by treatment with phenylglyoxal. The activity of the chemically modified ricin A chain in a rabbit reticulocyte lysate was strongly inhibited by this treatment and could be reversed by treatment with NH_2OH which removes the phenylglyoxal. Previously, Srinivasan and colleagues had performed similar work with gelonin (Srinivasan et al., 1985) and had also shown that chemical modification of a number of other amino acids, including lysine, did not reduce biological activity. Furthermore, the modification of the arginine residues did not induce gross changes in the conformation of gelonin. Funatsu showed that it was the arginine residues at the N - terminal part of the molecule that were involved in this activity. Sequence comparison with trichosanthin shows that three of the N - terminal arginine residues implicated by the chemical modification studies of Funatsu and co - workers were conserved (residues 29, 48 and 56 in ricin A chain) and may therefore be involved in the enzymatic reaction. Arginine residues appear to play an important role in the active sites of many enzymes that act on negatively charged substrates. In the case of the toxin A chains, it is therefore possible that this site interacts with the RNA in the 60S ribosomal subunit. Furthermore, arginines 48 and 56 appear to lie in the cleft proposed as the putative active site of ricin A chain

(Montfort et al., 1986) and would thus be excellent choices for DNA manipulations by site directed mutagenesis.

Figure 1.5.1 shows amino acid sequence alignments for all the RIP sequences available to date. In particular it should be noted that sequence homology is particularly strong in the regions SEAAR, GYRAG and LREWI and that arginine 56 on the ricin A chain sequence can be aligned with the corresponding residue in abrin too. A sequence showing strong homology with shiga like toxin A (SLT A), at the C - terminus of ricin A chain (not shown in figure 1.5.1) could also represent a shared function. Ricin A chain and SLT A are both internalised into the cytosol of eukaryotic cells by a similar mechanism; a speculated interaction with the relevant B chains. It could be that the amino acids in this similar sequence have a critical role in the interaction with the B chain during the internalisation of the A chains. Trichosanthin, which is internalised by a completely different and unknown mechanism, does not have this C - terminal extension. The selective uptake of trichosanthin by fetal trophoblastic cells is particularly interesting since the protein is not glycosylated, nor does it have an associated B chain.

Taxonomically, the proteins showing such striking sequence homologies are unrelated. In particular, the finding of homology between SLT A and ricin A chain is particularly intriguing because it crosses the boundary between prokaryotic and eukaryotic organisms. Several observations suggest this homology is significant; they both share similar mechanisms of activity and internalisation and predictions of secondary structure were virtually identical in the regions showing the most significant homology. (Calderwood et al., 1987). The debate as to whether the ricin B chain is a modern version of an extremely ancient precursor could very easily be applied

Figure 1.5.1. Alignment of homologous amino acids in the A subunits of Shiga - like toxin I (Slt-IA), ricin A chain, trichosanthin and abrin. Conserved amino acids are enclosed in boxes. Numbers in parentheses refer to the positions of residues in the mature protein. Dashes indicate gaps introduced into the sequences to maximise alignments. (Slt-IA: De Grandis et al., 1987; Abrin: Funatsu et al., 1988; Trichosanthin: Xuejun and Jiahuai, 1986). Aligned sequences above represent the sequences including Arg 48 and 56, of ricin A chain, investigated in chapter 8, sequence LRENI and sequence GYRAG, both mentioned in the text. The functional significance of sequence VTNAY, (1.5.1.2.) of ricin A chain is investigated in chapter 7. Sequence SEAAR, investigated in chapter 8, is shown in figure 10.3.

to the possible evolutionary scenario concerning the toxic A chains and could go some way to explaining why they are so universally ubiquitous. To extend this argument it is very tempting to suggest that the strong sequence homologies are a result of the fact that during the course of evolution a protein evolved that did its job so well that functional divergence was obsolete. The modern A chain toxins may be very closely related to their distant ancestors as demonstrated by their distribution from prokaryotes to higher plants. Even when the toxin gene fused with the lectin B chain gene functional diversification was unnecessary and as speculated earlier, during the process of this fusion the protein changed from having a passive role in defence (one chain toxins) and adopted an aggressive role as seen in the two - chain toxins.

A further sequence shared by abrin, ricin and trichosanthin is the sequence DVTHAY on the ricin A chain sequence at residues 75 - 80. The possible functional significance of this sequence is explored in detail in chapter 6.

1.5.2. Expression of RIP genes in heterologous systems.

The expression of the cloned toxin genes in heterologous systems has been an important recent development. Ricin A chain (O'Hare et al., 1986; Robertus et al., 1987) and Pseudomonas exotoxin (Gray et al., 1985) have both been successfully expressed in E. coli. The expressed protein can be highly purified and shows the same toxicity as the native protein. Ricin A chain expressed in E. coli is non - glycosylated, it is expected that such A chain used in vivo will not be rapidly cleared by mannose and fucose receptors on reticuloendothelial cells. It is predicted that immunotoxins

constructed using such protein will consequently have a longer half life during in vivo cancer therapy(see section 1:6:3:6). Attempts to express proricin or ricin A chain in yeast on inducible plasmids have been thwarted by the extreme toxicity of the toxin to this system. currently expression from DNA integrated within the chromosomes is being attempted.

The expression of ricin B chain in yeast (Richardson et al., 1987) and Xenopus oocytes (Richardson et al., 1987) has been achieved although attempts to express the protein in E. coli. have been hampered by the low solubility of the expressed product. It is envisaged that the expression of non - glycosylated ricin B chain in E. coli will provide material for the construction of a new generation of immunotoxins with a much higher degree of cytotoxicity to the target cells. (see section 1:6:3:6). Furthermore identification of the amino acids responsible for cell binding and their subsequent manipulation will enable the manufacture of modified toxins which retain the ability to insert into the membranes and transfer the A chain into the cytosol, yet which no longer bind non - specifically to cells.

1:5:3. Understanding the interaction of RIP's and the ribosome.

It had been speculated for a number of years that the site of action of the ribosome inactivating proteins was either the ribosomal RNA or one of the many proteins associated with the ribosome. Two observations supported this view;

1. It has been known for a number of years that ricin A chain binds dinucleotides

2. More recently, Watanabe and Funatsu (1986), showed that the chemical modification of specific Arg residues in ricin A chain and

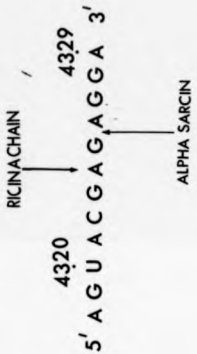


FIGURE 1.5.2 THE SITE OF ACTION OF RICIN A CHAIN AND ALPHA SARCIN ON 28S rRNA.

Taken from Endo et al., 1987

gelonin reduced the activity of the protein and that this was not caused by a gross change in the conformation of the protein. This suggested that the substrate was negatively charged and heightened the speculation that it was in fact the ribosomal RNA.

The catalytic and irreversible nature of the inactivation of 60S ribosomal subunits and the fact that no cofactor is required, led to the suspicion that ricin has endonuclease activty, like two other ribosome inactivating proteins, colicin E3 and sarcin. Despite many attempts, nucleolytic activity of ricin A chain was never convincingly demonstrated. (Mitchell et al., 1976).

Now, work by Endo, Tsurugi and co - workers demonstrates that the A chain of ricin is a highly specific N - glycosidase that removes a single adenine residue from the 28S RNA of the 60S ribosomal subunit.(Endo et al., 1987; Endo and Tsurugi, 1987). Their studies of the effect of ricin on rat liver ribosomes, showed that ricin modifies rat 28S rRNA in the region of G_{4323} and A_{4324}. (Endo et al., 1987). Furthermore, this site of modification is very close to the point at which alpha sarcin cleaves the rRNA. (Endo and Wool,1982; Chan et al.,1983) (see figure 1.5.2). They then went on to show that the effect of ricin on the 28S rRNA was to remove A_{4324} by N - glycosidic cleavage,thus rendering the phosphodiester backbone hypersensitive to cleavage by alkali and aniline at low pH. Similar activity on 28S rRNA is also exhibited by abrin and modeccin, suggesting a general mechanistic pathway for ribosome inactivation by toxic lectins.

Interestingly, A_{4324} lies in a region that is strongly conserved between species, apparently sticking out from the bulk of the ribosome as a hairpin loop. (Veldman et al., 1981; Hogan et al., 1984). Prokaryotes, such as Escherichia coli, also possess this conserved loop. However their ribosomes are resistant to the action of ricin and

related toxins, similarly, Endo and colleagues found that ricin had no N - glycosidase activity on such ribosomes. (Endo et al., 1987). This led to speculation that ricin A chain recognises the 3 - D structure around A4324, which is presumably determined by the association of theRNA with the ribosomal proteins, which differ between prokaryotes and eukaryotes. Endo also reported that ricin A chain could depurinate naked 28S rRNA free from the associated ribosomal proteins. However, the rate and the amount of modification of the naked RNA was slow and far less than the RNA present in the ribosomal particle. It was proposed that this was due to the fact that the isolated rRNA had lost some of the native secondary structure resulting in less than quantitative modification of the rRNA. Although this was further support for the theory that the ribosomal proteins play an important role in the interaction of ricin A chain and the 28S rRNA, it has so far been impossible to repeat these results in this lab. Despite numerous attempts, using high concentrations of ricin A chain for extended incubations with naked RNA isolated from both yeast and rabbit reticulocytes, Dr. Martin Hartley has been unable to achieve cleavage of naked 28S rRNA by ricin A chain and aniline treatment. Recently published work by Endo and colleagues (Terao et al., 1988), has added more weight to the theory that the involvement of the ribosomal proteins in the interaction of ricin A chain and 28S rRNA. They showed that both ricin and alpha sarcin, despite the fact that they have differing mechanisms of action (N - glycosidase and ribonuclease, respectively), alter the conformation of 60S ribosomal subunits at neighbouring, but different sites. It was found that labelling of protein L14 was specifically reduced by treatment with ricin, and that of proteins L3 and L4 with alpha sarcin, suggesting that the toxins alter the conformation of ribosomes in the vicinity of

these proteins (Terao et al., 1988). Recent evidence that rRNA's play important roles in protein biosynthesis (Moller, 1984), suggests that cleavage or modification of 28S rRNA by these two toxins directly lowers the protein synthesising activity of ribosomes as proposed in the case of alpha sarcin (Endo and Wool, 1982). It must be noted, however, that conformational changes may be important in the inhibition of ribosome activity by these two toxins, since high concentrations of Mg^{++} restore the activity of toxin - treated ribosomes (Cawley et al., 1979). Of the three ribosomal proteins which display altered conformation after treatment of ribosomes with ricin or alpha sarcin two, namely L3 and L4, appear to play important roles in protein biosynthesis. L4 is known to be located at the A site and L3 and at the P site (Terao et al., 1988) furthermore, L3 and L4 can be crosslinked with EF 2
(Uchiumi et al., 1986) and these proteins are located at the boundary of the 60S and the 40S subunits(Uchiumi et al., 1986) and interact with mRNA (Takahashi and Ogata, 1981 and 1985). It was therefore proposed, that ricin A chain and alpha sarcin - induced changes in these regions could account for the effects of these toxins on the protein synthesising activity of ribosomes, such as their effects on EF 2 binding and GTP hydrolysis during peptide chain elongation.

More recently, Moller and colleagues (Moazed et al., 1988) presented evidence which strongly suggested that depurination of the 28S rRNA in the conserved loop prevents EF 1 and EF 2 binding to the 28S rRNA and therefore **directly** inhibits protein synthesis. This is in aggreement with earlier studies which showed that competitive binding of EF 2 to ribosomes prevented their inactivation by ricin A chain.

(Fernandez - Puentes et al., 1976). Furthermore, as EF 2 and EF G are involved in ribosome - dependent GTPase functions in eukaryotes and prokaryotes respectively, the results of Moller and colleagues suggest a specific function for the universally conserved loop of ribosomal RNA.

Subsequently to the work of Endo and colleagues it has been found that N - glycosylating activity is universal of ribosome inactivating proteins so far studied; tritin, momordin, saporin, barley inhibitor, dianthins, dodecandrin, gelonin, PAP, trichosanthin and volkensin (Stirpe et al., 1988) and viscumin (Endo et al., 1988) all, not surprisingly, showing the same activity. Remarkably, the bacterial shiga toxin and Vero toxin from E. coli O157:H7 (shiga - like toxin II) both had exactly the same mechanism of action as the plant toxins and removed A_{4324} from the 28S rRNA of rabbit reticulocyte and rat liver ribosomes. As described above the recently published sequence of shiga - like toxin shares much homology with the primary sequence of ricin A chain the fact that they share exactly the same mechanism of action underlines the possible evolutionary link between the two proteins.

SECTION 1:6. APPLICATIONS IN CANCER THERAPY: IMMUNOTOXINS.

The idea of using antibodies directed against tumour associated antigens to target a toxic substance specifically to tumour cells has appealing simplicity. The antibody should cause the toxic agent to concentrate within the tumour tissue and so spare normal tissue from harm. Several different toxins, including ricin, abrin, gelonin, saporin and pokeweed antiviral protein, from plant sources and the bacterial toxins diphtheria toxin and Pseudomonas exotoxin A have been coupled to antibodies to produce 'immunotoxins'. The immunotoxins (IT's), so produced have all, to a greater or lesser extent, been capable of specific cytotoxic effects on tumour cells in vivo and in vitro. (see reviews; Thorpe, 1985; Blakey et al., 1987; Vitetta and Uhr, 1985; Pastan et al., 1986). Ricin is the only toxin so far to have been used clinically as an IT, it is however, prototypic of the other toxins, and many of the resul'ts parallel those obtained with the other toxins. One of the aims of this present study was to provide material for the generation of novel recombinant immunotoxins; it is therefore necessary to review their structure and activity in some detail

1:6:1. The preparation of immunotoxins.

The linkage used to join the antibody and the toxin component must meet three criteria:

1. it should not impair the antigen - binding capacity of the antibody; in practice, antigen binding is generally unaffected by the introduction of only one to two crosslinking groups per antibody molecule

2. it must allow the active A chain component to enter the cytosol and kill the cell; this is thought to require release of the toxic component from the antibody carrier or, in the case of IT's containing intact toxin, from the B chain.

3. for in vivo therapy, the linkage must be stable enough to remain intact while the IT is passing through the tissues of the animal to its intended site of action.

The methods for generating linkages with these properties are different for IT's containing intact toxins and IT's containing isolated toxin A chains or single chain RIP's. Detailed methods for the preparation and purification of IT's have been described previously (Thorpe and Ross, 1982; Cumber et al., 1985; Wawrzynczak and Thorpe, 1987)

1:6:1:1 Immunotoxins containing isolated toxin A chains.

A chain IT's (IT - A's), are prepared by linking the A chain to the antibody or antibody fragment by means of a reducible disulphide bond. Such IT's often possess a cytotoxicity to target cells that approaches that of the toxin itself. A disulphide linkage is used because liberation of the free A chain by reduction inside the cell appears to be necessary for maximal cytotoxicity. In contrast, IT's in which the A chain is attached to the antibody via a thioether bond are usually much less toxic than those made with disulphide linkages (Jansen et al., 1982; Masuho et al., 1982; Ramakrishnan and Houston, 1984) suggesting that the splitting of the disulphide bond is required for the toxic component to gain access to the cytosol.

1:6:1:2 **Immunotoxins containing ribosome inactivating proteins**

As with IT - A's, RIP IT's in which the RIP is linked to the antibody via a disulphide bond show the highest cytotoxic activity. Since RIP's do not contain a free thiol group, one must first be introduced using a thiolating reagent such as 2 iminothiolane. Thereafter the procedures are similar to those used to prepare IT - A's. However, chemical derivatisation of the RIP may affect its ribosome inactivating activity depending on the type of crosslinker employed. For example, thiolation of gelonin by treatment with N - succinimidyl - 3 -(2 - pyridyl - dithio) propionate (SPDP) followed by reduction with dithiothreitol reduced its inhibitory activity on protein synthesis in rabbit reticulocytes by 10 - fold whereas thiolation with 2 - iminothiolane had no apparent effect (G. Clements, this lab, and Lambert et al., 1985). This problem does not arise withA chain IT's because the native A chain is released by reduction irrespective of the type of cross - linker used.

1:6:1:3 **Immunotoxins containing intact toxins**

Intact ricin IT's (IT - R's) are prepared by introducing a free thiol group into the antibody and an alkylating function into the ricin. The thiol and alkylating groups then react to produce an IT in which the toxin is attached to the antibody via a thioether linkage. This linkage is stable to reduction and precludes dissociation of the intact toxin from the antibody. However, such IT's are cytotoxic because they retain the natural disulphide bond between the A and B chain which is reduced once the A chain is in the reducing environment of the cytosol. The B chain presumably also is of some benefit in such constructs in aiding the entry of the A chain into the cell.

1.6.2. In vitro cytotoxicity.

1.6.2.1 Intact toxin immunotoxins

The first highly potent and specific IT was prepared by linking diphtheria toxin (DT) to horse anti - human lymphocyte globulin using a novel heterobifunctional coupling reagent (Moolten et al., 1975). The IT was over 1000 times more toxic to human lymphoblastoid cells than was free DT or a control IT made with normal horse IgG. It reduced the rate of protein synthesis of the cells by 50% at a concentration of about 10^{-11}M which is very low considering that this was done in the days before monoclonal antibodies and only about 2% of the antibody preparation could bind to the cells.

Since these initial reports, a wide variety of monoclonal and polyclonal antibodies have been attached to intact DT, PA, ricin and abrin and tested for cytotoxicity (Thorpe, 1985; Edwards and McIntosh, 1986).

IT's prepared using intact toxins are almost always extremely potent cytotoxic agents in vitro for cells with the appropriate target antigen, often surpassing the native toxin in potency. Predictably, however, they suffer from a lack of complete specificity because they can also bind to non - target cells via the cell binding domain of the B chain of the toxin. The non - specific binding of intact toxin IT's can be blocked in vitro by adding high concentrations of free lactose or galactose, without reducing he toxicity to target cells. (Youle and Neville, 1980; Houston and Nowinski, 1981; Thorpe et al., 1982; Thorpe et al., 1981). However, if the IT's are to be used in vivo, blockade of non - specific binding in this way is of little value since in animals such sugars are rapidly excreted. A more permanent blockade was reported by Thorpe and co - workers (Thorpe et al., 1984). They

linked monoclonal anti - Thy 1.1 antibody to ricin using a short crosslinker and found that much of the IT preparation was unable to bind to galactose residues probably because of steric hinderance of the B chain binding site by the antibody. This 'blocked' ricin IT was about 10,000 times more toxic to Thy 1.1 expressing AKR - A lymphoma cells than it was to EL4 lymphoma cells which express the alternative Thy 1.2 allele (Thorpe et al., 1984).

1:6:2:2 A chain immunotoxins

A large number of IT's have been made by linking the A chains of DT, ricin and abrin to polyclonal and monoclonal antibodies and these have recently been reviewed (Thorpe, 1985; Edwards and McIntosh, 1986). Two general findings have emerged.

1. A chain type IT's have been found to show virtually complete specificity in their cytotoxic effects upon target cells in vitro.

2. They have variable cytotoxic potency. Some A chain type IT's are as toxic to cells as their intact toxin counterparts whereas others are only weakly cytotoxic. This was clearly demonstrated in a recent study in which 85 monoclonal antibodies recognising breast cancer selective antigens were linked to ricin A chain and tested for cytotoxicity against four human breast cancer cell lines (Bjorn et al., 1985). Only 28% of the IT's were considered to be cytotoxic (ie., had IC50 values below 10nM).

1:6:2:3 RIP immunotoxins

Thorpe and co - workers demonstrated that gelonin acquired potent and specific cytotoxic activity after its linkage to monoclonal anti Thy 1.1 antibody (Thorpe et al., 1981a). Similar results have been reported since for other IT's made with the RIP's gelonin

(Colombatti et al., 1983; Wiels et al., 1984), PAP (Masuho et al., 1982a; Ramakrishnan and Houston, 1984a) and saporin (Thorpe et al., 1985). These IT's have similar potency and specificity to ricin A chain IT's prepared from the same antibody. The advantage of using the RIP's over the toxin A chains is that they are safer to handle in quantity and do not need the same rigorous purification to exclude traces of the intact toxin or B chain.

1.6.3. Ways to improve the cytotoxicity of an immunotoxin

1.6.3.1 Potentiation using lysosomotrophic amines and carboxylic ionophores.

Several authors have shown that various lysosomotrophic amines (Ramakrishnan and Houston, 1984a; Fulton et al., 1986; Jansen et al., 1984; Kronke et al., 1986; Casellas et al., 1984) and carboxylic ionophores (Raso and Basala, 1984; Casellas et al., 1984) can enhance the cytotoxic potency of weakly cytotoxic A chain IT's. These compounds accelerate the inhibition of protein synthesis and greatly reduce the number of IT molecules per cell required for cytotoxicity. It is possible that monensin and the lysosomotrophic amines disrupt the movement of the IT between different intracellular compartments, perhaps keeping it for longer in peripheral endosomes or some other compartment which favours escape into the cytosol. (Carriere et al., 1984). It remains to be seen whether effective concentrations of lysosomotrophic amines or carboxylic ionophores can be achieved in vivo to enhance the potency of weakly cytotoxic IT's without causing prohibitive toxicity.

1.6.3.2 **Potentiation using ricin B chain.**

The potency of IT's can be enhanced, sometimes drastically, by the addition of free B chain in the presence of lactose (McIntosh et al., 1983). Similarly, ricin B chain coupled to antibody can potentiate A chain immunotoxins in vitro. Vitetta and her colleagues showed that the specific toxicity of an A chain IT could be markedly enhanced by a 'piggyback' treatment with a B chain immunotoxin. (see figure 1.6.1.) (Vitetta et al., 1984), similar potentiation was obtained when the A and B chains were each linked to an anti human immunoglobulin antibody and targeted separately to the cells (Vitetta et al., 1983). The delivery of free B chain and B chain IT's to target cells in vivo is likely to be limited by their binding to galactose residues on blood and tissue glycoproteins and glycolipids. T circumvent such problems, Vitetta prepared a chemically modified B chain which had a 100 - 200 fold decreased ability to bind to the galactose terminating glycoprotein asialofetuin. The modified B chain was capable of potentiating A chain cytotoxicity in 'piggyback' assays although its effectiveness was slightly reduced. This work suggests that, at least in this test system, the galactose binding and potentiating functions of the B chain may be distinct. It is possible in these situations that free B chain aggregates to form a pore through which the A chain passes to the cytosol, or that the carbohydrate on the B chain directs the IT to a cellular compartment favouring A chain escape.

1.6.3.3 **Potentiation using viruses.**

Adenovirus has been used to potentiate the cytotoxic effects of PA coupled to epidermal growth factor (Fitzgerald et al., 1983a

and b) and PA or ricin A chain coupled to anti - transferrin receptor antibody (Fitzgerald et al., 1986). It appears that this occurs because the adenovirus and the IT entered the cells in the same intracellular vesicles and that the adenovirus disrupted the vesicles, permitting the entry of the IT into the cytosol. The membrane - disrupting component of the adenovirus appears to be a capsid protein (Fitzgerald et al., 1986). It may therefore be possible to isolate this protein and use it to potentiate IT's in an analogous fashion to that described for B chain.

1.6.4. Anti-tumour activity of ITs in vivo.

1.6.4.1 Results in experimental animals

One of the best anti-tumour effects to date with an IT was reported by Krolick and his co - workers (Krolick et al., 1982). Mice bearing massive B cell leukaemia burdens (20% of body weight, or approximately 10^{10} tumour cells) were treated with cytoreductive therapy (splenectomy and total lymphoid irradiation) followed by intravenous administration of an IT which binds to surface IgD on the tumour cells. This treatment rendered the animals free from disease whereas animals given cytoreductive therapy followed by a control IT or by free anti - antibody developed leukaemia. The disease-free animals still harboured dormant tumour cells but this shows that IT's need not kill every tumour cell in vivo for prolongued remissions to occur, since a few remaining tumour cells may be held permanently in check, probably by the immune system. Since this and other reports, anti-tumour experiments have been conducted using IT's containing ricin A chain (Embleton et al 1986; Seto et al., 1982; Kishida et al., 1983) gelonin (Thorpe et al., 1981a), PAP (Ramakrishnan and Houston,

1984b) or PA (Fitzgerald et al., 1986). Generally, though, the first attempts at therapy with these IT's produced less impressive results than it was hoped for. Differences observed between in vitro and in vivo effects were sometimes striking (Thorpe et al., 1985), this difference strongly indicated that early IT's containing toxin A chains were subject to problems in vivo which reduced their therapeutic effectiveness. Furthermore these results indicated that the ability of an IT to reduce protein synthesis in target cells in vitro can be a poor indicator of its anti-tumour activity in vivo.

1:6:4:2 Problems of in vivo targeting.

1. Recognition by cells in the liver.

To be effective in vivo, IT's must remain in an active form in the bloodstream for long enough for a therapeutic dose to reach the tumour site. It is now clear that IT's containing ricin A chain are cleared much more rapidly from the bloodstream of animals than the parent antibody (Jansen et al., 1984; Raso and Basala, 1984). The rapid clearance of ricin A chain IT's is due primarily to the recognition of mannose and fucose residues on the A chain by cells with mannose or fucose receptors in the liver and other organs. (Bourrie et al., 1986; Thorpe et al., 1985a; Skilleter et al., 1986). This problem can be overcome either by chemically modifying the carbohydrate on ricin (Thorpe et al., 1985a) or by removing the carbohydrate enzymically (Skilleter et al., 1986). IT's made with deglycosylated ricin A chain are cleared much less rapidly than their native counterparts in mice (Blakey et al., 1987), although these IT's are still cleared faster than the parent antibody. Increased blood clearance rates are also seen with IT's containing PAP (Ramakrishnan

and Houston, 1985), saporin (Letvin et al., 1986) both of which are naturally devoid of carbohydrate. An obstacle to the use of ricin B chain IT's for potentiating the activity of ricin A chain IT's in vivo is that, as with ricin A chain, the mannose - terminating oligosaccharides present on the B chain will be recognised by the cells of the liver and cause the rapid clearance of the B chain IT from the bloodstream. Chemical deglycosylation of B chain only slightly reduces the ability of B chain IT's to potentiate the cytotoxicity of ricin A chain in 'piggyback' experiments (Vitetta and Thorpe, 1985). Deglycosylated B chain IT's should therefore give better potentiation of A chain IT's in vivo.

2. Linkage instability

The disulphide bond between the antibody and toxin introduced by the heterobifunctional coupling reagents SPDP or 2 - iminothiolane is at least in some cases, unstable in vivo. Thorpe and co - workers have found that anti - Thy 1.1 ricin A chain and abrin A chain IT's consistently break down slowly in mice to release free antibody (Blakey et al., 1987,). Instability of IT's not only reduces the amount of conjugate available for therapy but also results in the release of free antibody which can compete with the IT for tumour-associated antigens and which, being longer lived, has a greater opportunity to bind to them. The stability of A chain IT's can be greatly improved without reducing their cytotoxic potency by using new cross - linking reagents that introduce hindered disulphide bonds which are more resistant to reduction (Worrell et al., 1986).

3. Tumour cell mutants

The presence of subpopulations of tumour cells that are resistant to IT's is certain to pose a problem for IT therapy in vivo as indeed it does with all types of chemotherapy. Heterogeneity of antigen expression has been observed with many types of tumour cells (Woodruff, 1983) and so is almost certain to be a routine problem with IT therapy. It may be possible to overcome this problem by using a cocktail of IT's directed against a variety of tumour associated antigens provided that the lesion that prevents the expression of one antigen does not also prevent the expression of the others. Tumour cell mutants may also emerge which are resistant to the action of the toxin. If so, it may be necessary to use a cocktail of IT's made from toxic components whose mode of action does not overlap.

4. Cross reactivity

Cross reaction of the antibody component with antigens on normal tissues may lead to normal tissue damage during IT therapy. Nevertheless, antibodies that react with normal tissues can still be used for therapy in a number of situations. For example, if the tissue is not life sustaining, such as lymphoid cells that are rapidly replaced by new cells which emerge from the bone marrow. Another instance is where the normal tissue may express the target antigen at a lower density than the malignant cells and so escape damage.

5. Antigen shedding

Another potential problem for IT therapy in vivo is that the target antigen may be shed either naturally or as a result of target cell killing. This would reduce the amount of IT capable of reaching and binding to the target cells, and cause toxicity to the liver, spleen

and other organs responsible for clearing the IT - antigen complexes. It may therefore be necessary to remove circulating target antigen before IT therapy by plasmaphoresis.

6. Immunogenicity

Injection of IT's is likely to lead to an immune response against both the antibody and the toxin portion of the IT. As with the presence of the target antigen in the serum, this may cause damage to the organs that clear the complexes and reduce the plasma half - life of the IT. The induction of neutralising antibodies in patients treated with mouse monoclonal antibodies is well documented (Levy and Miller, 1983). The use of human monoclonal antibodies may reduce this problem although even these are likely to evoke an anti - idiotipyic immune response. If repeated injection of IT's is required for therapy, it may be necessary to immuno - suppress the patients or change the antibody and toxin portion of the IT from time to time.

7. Access in solid tumours

Perhaps the biggest obstacle facing the use of IT's to treat solid tumours is that the IT's will not have sufficient access to the cancer cells to be effective. Access to solid tumours might be improved by the use of antibody fragments or by co - administering a vaso - dilating agent. Alternatively it might be possible to target immunotoxins to the vasculature of a solid tumour and so kill the tumour cells indirectly by starving them of nutrients.

1:6:4:3 Immunotoxin therapy; clinical trials.

So far, there have been three reports describing the in vivo clinical use of immunotoxins in man. (Blakey and Thorpe, 1988). Three diseases were treated; advanced metastatic melanoma, T - cell

acute lymphoblastic leukaemia and steroid-resistant graft versus host disease. The results were encouraging in that remission of the symptoms occured in several of the patients suffering from the diseases treated with the ricin A chain immunotoxin. Encouraging though these results were, it must be stressed that the IT's used in these clinical trials were prepared by linking native A chain to antibody by means of the SPDP reagent and that the IT's were not purified to remove free antibody. The IT's would be expected to suffer from the same problems in man as they did in experimental animals as described above. It is envisaged that, in the future treatment of the above and other diseases (see Pastan et al., 1986, for a review) will continue but that a new generation of immunotoxins will be employed, namely immunotoxins prepared using recombinant material, as described below.

1.05. Production of a new generation of ITs by recombinant DNA technology.

It is envisaged that by employing cloned toxin genes for the construction of a new generation of immunotoxins a more rational approach to solving the problems of immunotoxin therapy in vivo may be achieved.

The genes encoding DT (Greenfield et al., 1983; Leong et al., 1983), PA (Gray et al., 1984) and preproricin, the precursor of ricin, (Lamb et al., 1985; Halling et al., 1985) have been isolated and characterised.

IT's have been constructed by chemically coupling recombinant products of the DT gene expressed in E. coli to the UCHT1 monoclonal antibody. (Colombatti et al., 1986). Other recombinant immunotoxins

have been developed. The discrete domain structure of PA was revealed by X - Ray crystallography (Allured *et al.*, 1986), by deleting distinct portions of the gene cloned in expression plasmids the synthesis of different functional polypeptides was achieved (Hwang *et al.*, 1987). The elimination of part of the toxin domain 1, which is involved with cell binding, yielded a product that was 200 - fold less toxic to mice than was native PA. More recently, a recombinant fusion protein was constructed by fusing the genes encoding transforming growth factor type alpha and domain III of PA and expressed in *E. coli*. (Chaudhary *et al.*, 1987). The fusion protein was effective in killing cells expressing epidermal growth factor and had little activity against cells with few receptors. It is envisaged that the protein might be useful in treating cancer cells that have high numbers of epidermal growth factor receptors.

It is now generally accepted that the development of recombinant IT's will be the best approach to solving the problems of cancer therapy *in vivo* for the following reasons:

1. By constructing the IT as a gene fusion between an antibody gene and a toxin gene one evades the problems associated with:

a) chemical derivatisation causing toxin inactivation and linkage instability

b) dissociation of the toxin and the antibody releasing free antibody which competes with the IT for receptors and (in the case of two chain toxin IT's) free toxin which may cause non specific cell death.

c) purity of the immunotoxin. By expressing the fusion protein in *E. coli* highly purified IT can be synthesised away from contaminating free antibody and toxin.

2. By expression of recombinant IT's in E. coli, the toxin will not be glycosylated and this will thus prevent non-specific binding of whole toxin or B chain (for potentiation purposes) and will increase the half-life of the toxin by preventing uptake of the A chain by mannose receptors on reticuloendothelial cells. (Thorpe et al., 1985a).

3. By identifying the active site of the toxin and subsequent manipulation of the cloned gene an A chain of reduced size could be expressed to reduce its immunogenicity and facilitate accessibility to tumours. An A chain of minimum genetic structure should, however, exhibit full catalytic activity.

Until the B chain galactose-binding sites have been permanently eliminated, safety and ethical considerations will dictate the continued application of A chain immunotoxins, particularly for use in vivo. In order to develop the most powerful immunotoxins it is essential to include the B chain in the conjugate. To eliminate or effectively reduce the ability of B chain to bind galactose, it is necessary to identify the amino acids involved and to introduce appropriate base changes in the encoding DNA which can then be expressed in a heterologous, non - glycosylating system. The predictions made from the X - ray structure of ricin as to the amino acids involved in galactose-binding are being currently explored by several groups. It is envisaged that B chain could then be expressed which retains the ability to insert into membranes and transfer the A chain into the cytosol but which no longer binds non - specifically to cells. It may also be possible using such approaches to identify the most immunogenic stretches of the A and B chains and to delete the DNA encoding these sequences from the gene.

Another goal is to produce immunotoxins entirely by recombinant DNA technology. Transfection of myeloma cells with immunoglobulin

genes ligated to toxin genes could facilitate the production of large quantities of immunotoxins provided that mutant cell lines resistant to ricin A chain activity were employed. The feasibility of this approach has been demonstrated by Neuberger and his colleagues for monoclonal anti-hapten antibodies ligated to staphylococcal nuclease. (Neuberger, 1985). The linkage of the A chain to an antibody via conventional DNA splicing methods might prove problematic since the A chain and the antibody must be cleaved in the endosome. However, attempts are underway to ligate these genes with an intervening sequence encoding a polypeptide that can be split by proteolytic enzymes (eg. the Arginine rich region of diphtheria toxin) inside the target cell or associated with its surface. Work is also underway to prepare an A chain linked to the antigen-combining site of the antibody via the Arg - rich portion of diphtheria toxin.

SECTION 1:7. AIMS OF THE PROJECT.

Ricin A chain is the plant ribosome inactivating protein most frequently used for the construction of immunotoxins. Interest in these conjugates is based on their potential as therapeutic agents which could be selectively targeted to destroy neoplastic cells or subsets of lymphocytes (for review, see section 1:6). Problems which arise when such reagents are used in in vivo therapy include;

1. The development of a host - antibody response against both the monoclonal antibody and the toxin.

2. Lack of penetration of the IT into a solid tumour.

It is generally recognised that the construction of immunotoxins containing recombinant toxins will represent a significant contribution to improving their in vivo toxicity and specificity. In an effort to achieve such goals, it is desirable to delineate the smallest possible A chain fragment which still retains full catalytically. One of the aims of this present study was therefore, by progressive deletion of the 5' or 3' terminus of the A chain gene, to determine the minimum genetic structure which still retains full catalytic activity. Such a DNA fragment, when cloned in an E. coli expression vector could be synthesised in large quantities and used in the construction of novel immunotoxins. It is envisaged that such a molecule, because of its reduced size, would be able to penetrate solid tumours with more ease than would a larger molecule and similarly, would possibly show a lower host antibody response in vivo and thereby show slower clearance from the bloodstream.

A long term goal of many groups working in this area is to understand the molecular details of the mechanism of catalytic inhibition of protein synthesis by plant ribosome inactivating

proteins. To this end, using the cloned ricin A chain sequence and comparison with other, related toxin sequences and the X - ray structure of ricin A chain site-directed mutagenesis would be used to modify specific bases or regions of the gene. The expression and assay of such mutants would shed light on the amino acids and regions of the protein which encode the toxic function, ribosome-binding site or regions involved in the interaction with the B chain during the transport of the A chain into the cytosol of eukaryotic cells.

Whist in preceding sections of this chapter much evidence has been presented as to the mechanism and nature of ricin A chain activity it should be stressed that at the outset of this work (1985), very little of this information was available. Indeed at this time, only the sequence of ricin A chain was known, and it was some time before the X - ray structure had been determined, (1987) or other sequences were available for comparison (1987 - trichosanthin). Furthermore, it was even later that details of the molecular action of ricin A chain on the ribosome emerged (1987 - 1988)

Conventionally, the functional analysis of cloned genes requires the expression of the wild type and manipulated gene in a heterologous or homologous expression system, purification and assay. At the outset of the work, this technology was not available, although, ricin A chain has subsequently been successfuly expressed in a soluble catalytically active form in E. coli (O'Hare et al., 1987). Furthermore the use of heterologous expression systems for the purposes proposed would be very time consuming. It was proposed that, in the absence of information concerning the 3D structure of ricin (which was due to appear at about that time), the primary objective was to develop a more rapid system for the analysis of the activity of cloned ricin A

chain. If and when details of the 3D structure of ricin and sequence information for other RIP's became available, this could be used to predict the regions of ricin A chain which should subsequently be mutated and the functional significance of this modification assessed.

The following chapters discuss the results from these lines of investigation. They report for the first time that fully functional protein can be expressed from in vitro synthesised RNA in a cell free system. The potential of this simple, rapid system to characterise modifications to the cDNA sequence is fully exploited in the light of information which became available during the course of this study. By employing this system, I describe preliminary experiments which indicate the putative ribosome binding-domain of ricin A chain. Work also undertaken using this system indicates where attention in modifying the sequence should be focussed in the future for the identification of residues involved in the catalytic activity of the protein or for generating deleted sequences for novel immunotoxin construction.

CHAPTER 2.

MATERIALS AND METHODS.

SECTION 2:1. MATERIALS.

2:1:1 Chemicals, biochemicals, radiochemicals and enzymes.

All materials used were of the highest analytical grade available. The source of specific reagents is given below:

Amersham International PLC., Amersham, Buckinghamshire;

[γ-^{32}P] - ATP (180TBq/mMol), [α-^{35}S]dATP α S (44TBq/mMol), [α-^{32}P] UTP (30TBq/mMol), [5, 6 -^{3}H] UTP (1.92 TBq/mMol), L - [^{35}S] methionine (30TBq/mMol), DNA polymerase I (Klenow fragment), all restriction endonucleases, (unless otherwise stated), T4 DNA ligase, T4 DNA polymerase, T4 DNA kinase, SP6 RNA polymerase, T7 RNA polymerase, Rabbit reticulocyte lysate, supplemented and nuclease treated.

The Boehringer Corporation, (London), Ltd., Lewes, East Sussex;

CIP, rATP, rCTP, rGTP, rUTP, dATP, dCTP, dGTP, dTTP, ddATP, ddCTP, ddGTP, ddTTP, tRNA from bakers yeast, tRNA from wheatgerm, T7 RNA polymerase, nuclease (micrococcal nuclease from Staphylococcus aureus), Creatine phosphate, Creatine phosphokinase, SP6 - promoter - directed sequencing primer.

BDH Chemicals Ltd., Poole, Dorset;

Acids, Amberlite monobed resin MB - 3, Ammonium persulphate, Bromophenol blue, β - Mercaptoethanol, Acrylamide, Xylene cyanol, Polyethylene glycol, TEMED, Caesium chloride.

Fisons PLC., Loughborough, Leicestershire;

Acrylamide, Formamide, Formaldehyde, Glycerol.

Difco Laboratories, Basingstoke, Hampshire.

Bacto - agar, Bactotryptone, Yeast extract.

Eastman Kodak, Rochester, New - York;

Bis - Acrylamide.

Gibco - BRL, Paisley, Renfrewshire, Scotland;

Low melting point agarose (ultra - pure).

May and Baker Ltd., Dagenham, Kent;

Trichloroacetic acid.

New England Biolabs, Beverly, Massachusetts, USA.;

Exonuclease III

Northumbria Biologicals Ltd., Cramlington, Northumberland;

Restriction endonuclease Sph I.

Promega Biotec Ltd., Madison, Wisconsin, USA;

RNAsin, SP6 RNA polymerase.

Schleicher and Schull, Dassel, FRG;

Nitrocellulose type BA85/1.

Sigma Chemical Company Ltd., Poole, Dorset;

Agarose medium EEO (type II), Ampicillin (sodium salt), ATP, BSA, Chloramphenicol, DTT, EtBr, IPTG, Lysozyme, MOPS, RNAase A, (bovine pancreas), S1 Nuclease, Spermidine 3 - HCl, Spermine 3 - HCl, X - gal, Coomassie Brilliant Blue R, HEPES, Tris, L - amino acids, EGTA, EDTA, Trypsin, Thermolysin.

SECTION 2:2 STORAGE OF BIOLOGICAL MATERIALS.

2:2:1. Storage of bacterial stocks.

Short - term storage of strains was on either minimal agar plates (5.25g K_2HPO_4, 2.25g KH_2PO_4, 0.5g (NH_4)$_2SO_4$, 0.25g tri - sodium citrate, 1.25mg thiamine - HCl, 0.4g glucose, 0.25g $MgSO_4$ per liter sterile distilled water, solidified with bacto - agar to 1.5%); or LB agar plates (LB solidified with 1.5% bacto - agar). A loopful of a fresh overnight culture was spread onto a dried plate. Following incubation at 37° c overnight, plates were stored at 4° c for up to two weeks, then re - streaked.

2:2:2. Storage of M13 stocks.

M13 phage were stored either as frozen (-20°c) single - stranded DNA in sterile distilled water or as RF DNA at 4° c in TE (10mM Tris - HCl pH 7.5, 1mM EDTA).

SECTION 2:3 NUCLEIC ACID ISOLATION

2:3:1. Large scale plasmid DNA isolation.

Plasmids were isolated from E.coli. by a modification of the alkaline lysis method of Birnboim and Doly, (1979). Ten milliliters of LB, (10g bacto - tryptone, 5g yeast extract, 10g NaCl, pH 7.5 per liter of sterile distilled water) containing ampicillin (100µg / ml), in 25ml universal containers, were inoculated with single bacterial colonies from fresh LB agar plates (solidified with bacto - agar to 1.5%). Cultures were grown overnight in an orbital shaker at 200 rpm and 37° c. Two litre flasks containing one litre of LB plus 100µg / ml ampicillin were inoculated with the 10ml overnight cultures and grown at 37° c / 200 rpm to an A_{650} value of 0.8 units. Plasmid DNA was amplified by the addition of chloramphenicol to 175µg / ml. The flasks were shaken at 37° c for a further 16 hours. Cells were collected by centrifugation at 2500 rpm and 4° c for 30 min in an MSE 6 1000 rotor. The cells were resuspended in 3.2ml of ice - cold GET (50mM glucose, 10mM EDTA, 25mM Tris - HCl pH 8), transferred to 50ml MSE Oakridge centrifuge tubes and placed on ice. Cell lysis was achieved by the addition of 200µl GET containing lysozyme at 40mg / ml and incubation on ice for 10 minutes; 6.6ml of 0.2N NaOH, 0.1% (w/v), SDS was added to each tube and the tubes gently swirled. After 10 minutes on ice, 5ml of 3M sodium acetate pH 4.5 was added and the contents of the tube gently mixed. The tubes were left on ice for a

further 30 minutes. After this incubation the tubes were spun at 10,000 rpm for 15 minutes at 4° c in an MSE 8x50 rotor. Nucleic acid acid was precipitated by the addition of 8.3ml of isopropanol to the supernatants in sterile 50ml tubes. Following mixing, the tubes were left at room temperature for 10 minutes. The precipitates were pelleted by centrifugation at 10,000 rpm and 4°c for 10 minutes. The pellets were drained and resuspended thoroughly in 3.2ml 2M ammonium acetate and the samples respun at 10,000 rpm for 10 minutes as above. After centrifugation, 2.1ml isopropanol was added to each sample in sterile 50ml tubes. After 10 min. at room temperature the resulting precipitates were collected by centrifugation as above. The pellets were washed twice in 50ml 70% (v/v) aqueous ethanol. The plasmid pellets were gently dried in vacuo and resuspended in 20ml TE (10mM Tris - HCl pH8 / 1mM EDTA).

Plasmid DNA was further purified by CsCl density gradient centrifugation (Maniatis et al., 1982). CsCl (23.76g) was gently dissolved in each of the 20ml plasmid solutions. EtBr (4ml of 5mg / ml) was added to each sample and the resulting solutions loaded into 37ml Beckman Quickseal tubes. The tubes were topped up with liquid parafin, balanced and sealed. The gradients were formed by centrifugation in a Beckman Vti 50 rotor run at 45,000 rpm and 20° c for 18 hours. The rotor was brought to rest without the aid of a brake. Plasmid bands were visualised under long - range UV and withdrawn from the pierced tubes in a volume of under 2ml with an 18 guage needle. The EtBr was removed from the samples by several extractions of isopropanol saturated with CsCl saturated sterile

distilled water. The CsCl was removed from the samples by dialysis against several litres of TE. Dialysis tubing was prepared by boiling lengths of tubing in 250mM EDTA for 15 min., followed by several rinses in sterile distilled water. After 3 - 4 hours, the TE was changed and dialysis continued overnight. The plasmid DNA was stored at 4° c. A typical preparation yielded 500 - 1000µg DNA.

2.3.2 **Small scale plasmid preparation**

a). From a 10ml overnight culture of E.coli in LB - ampicillin, 1.5ml of cells were collected by centrifugation for 30 seconds in an Eppendorf centrifuge. Cell lysis was obtained by resuspension of the pellet in 80µl GET and 5µl 40ug / ml lysozyme on ice for 5 minutes; 160µl of 200mM NaOH, 1% (v/v) SDS was added and the tube vortexed. After five min. on ice 100µl of 3M NaOH pH 4.5 was added, mixed and stood on ice for a further 5 min. After this incubation, the tubes were spun for 5 min. in an eppendorf centrifuge, the clear supernatant removed and phenol / chloroform extracted as described in section 2.3.1. Nucleic acid was precipitated by incubation with two volumes of ethanol on ice for 20 min. followed by centrifugation for 30 min. Nucleic acid pellets were resuspended in 50µl TE (10 mM Tris - HCl pH 7.5, 1mM - EDTA).

b). From a 10ml overnight culture of E.coli in LB - ampicillin, 1.5ml of cells were collected by centrifugation for 20 sec. in an eppendorf centrifuge. Most of the supernatant was removed

leaving a trace in which to resuspend the cell pellet by vortexing. Cell lysis was obtained by the addition of 300µl of STET (8% w/v sucrose, 0.5% v/v Triton X - 100, 50mM EDTA pH 8, 10mM Tris - HCl pH8), and 25µl of 10mg / ml fresh lysozyme. After a few seconds, the suspension was briefly vortexed and boiled for 1 min. followed immediately by centrifugation for 15 min. The cell debris pellet was removed using a sterile toothpick and 5µl of 10mg / ml RNAase A was added. After incubation at room temperature for 10 min. the solution was phenol / chloroform extracted once. The top half of the aqueous phase was removed and added to 0.1 vol. of 3M sodium acetate and 2 vol. ethanol. After a 20 min. incubation at room temperature, the tubes were spun in a microfuge for 10 min., the pellet washed in 800µl of 70% aqueous ethanol, briefly dried in vacuo and resuspended in 50µl of sterile distilled water.

2.3.3. M13 phage single stranded DNA isolation.

(Schreier and Cortese, 1979)

A 1.5ml aliquot of LB containing a 1 / 100 dilution of an overnight culture of E.coli strain 71:18, or JM101 was placed in a sterile 25ml container. To this a single, well - isolated plaque, picked from a fresh plate was inoculated using a Gilson pipette. The inoculum was grown at 37° c and 300 rpm for 5½ - 6 hours. The culture was transferred to a 1.5ml Eppendorf tube and bacterial cells pelleted

by centrifugation for 5 min. The supernatant, containing the phage - particles was poured carefully into a second tube and 200µl of 20% (w/v) PEG 6000, 2.5M NaCl, added. Following mixing, the tube was left to stand at room temperature for 15 min. The phage precipitate was pelleted by centrifugation in a microcentrifuge for 10 min., the supernatant removed using a pipette and the pellet re - centrifuged briefly after which any PEG was removed using a drawn - out pipette attached to a vacuum line. The viral pellet was resuspended in 100µl TE and 100µl of phenol saturated with TE was added. The tube was vortexed, left at room temp. for 5 min., revortexed thoroughly and spun in a microcentrifuge for 1 min. All of the aqueous phase was removed to a fresh tube and 50µl of TE - phenol, 50ul of chloroform added, vortexed, incubated at room temp. for 5 min. revortexed thoroughly, and spun as before in a microcentrifuge. To the aqueous phase was added 500µl ether and after mixing, the top ether phase was removed and discarded. The single - stranded DNA was precipitated after the addition of 10µl of 3M sodium acetate pH 6 and 2 vols. of ethanol at - -20° c for 1 hour. The DNA was pelleted by spinning in a microcentrifuge at 4° c for 30 min., the pellet washed twice in 70% (v/v) aqueous ethanol. The pellet was gently dried in vacuo, resuspended in 20ul sterile distilled water and stored at -20° c.

2:3:4 **M13 phage replicative form DNA extraction.**

Competent cells of E.coli strain JM101, (Messing et al., 1981), were transformed with 20ng of M13 RF DNA or 1µl of ssDNA (see section 2:2:3.). After incubation at 37° c overnight, a single, well - isolated plaque was inoculated into 10ml LB containing a drop of E.coli strain JM101 grown the previous night. This was the " phage overnight."

A second 10ml culture of E.coli strain JM101 was grown, the " feeder cells ". Feeder cells, (1ml), were inoculated into 40ml of LB and grownto an A_{550} value of 0.5 - 0.6. To this, 1 / 100th vol. of the phage overnight was added and the culture grown at 37° c, 300 rpm for 4 hours. The RF DNA was isolated by the following method, which can also be used for the preparation of plasmid DNA. The 40ml culture was collected by centrifugation at 5,000 rpm for 5 min. Cell lysis was obtained by resuspending the pellet in 2 ml. of SET, (25mM Tris - HCl pH8, 10mM EDTA, 15% (w/v) sucrose) with lysozyme to a final concentration of 2mg / ml, and incubated on ice for 15 minutes. To this, 4mls. of 0.2N NaOH, 1% (w/v) SDS was added and mixed, and after 10 minutes on ice, 2.5ml. of 3M sodium acetate pH 4.6 was added and mixed gently. After an incubation of 15 min. on ice cell debris was collected by centrifugation at 15,000 rpm for 15 min. To the clear supernatant, in a fresh tube, was added 25µl of 1mg / ml RNAase A followed, after incubation at 37° c for 15 min., by a standard phenol / chloroform extraction (see section 2:3:1.). Nucleic acid was precipitated by adding 0.6 vols. of isopropanol, incubation at room temp. for 15 min. and centrifugation at 15,000 rpm for 10 min. The

nucleic acid pellet was resuspended in 0.6ml water, 0.2ml of 4M NaCl, 1ml of 13% (w/v) PEG6000. This solution was split between two eppendorf tubes incubated on ice for 1 hour and the DNA was precipitated by centrifugation for 10 min. Excess PEG was removed using a drawn - out pipette attached to a vacuum line, the pellet rinsed inethanol, dried briefly in vacuo then resuspended in and appropriate amount of sterile distilled water.

2.5.5. Standard phenol extraction of DNA.

Phenol was saturated with TE buffer (10mM Tris - HCl pH 8, 1mM EDTA), and stored at 4° c in the dark.

1. To a volume of DNA solution, 0.25 vols. phenol and 0.25 vols. chloroform were added and the well - sealed tube vortexed thoroughly. For eppendorfs, tubes were spun for 1 - 2 min., or for bigger volumes in a large centrifuge for 5 min. at approximately 5,000 rpm. Opaque oakridge tubes were used in all cases for larger volumes.

2. The top aqueous phase was removed to a clean tube.

3. For efficient extraction, the organic phase was re-extracted using 0.5 vol. TE, tubes were respun as before and the new aqueous phase combined with the first.

4. Steps 1. was repeated.

5. The pooled aqueous phases were extracted with chloroform by adding an equal volume of chloroform, vortexing and spinning as before.

6. The aqueous phase was removed, and DNA concentrated from it by ethanol precipitation.

2:3:6. **Ethanol precipitation.**

The pooled aqueous phases from phenol / chloroform extractions or any large volumes of DNA in aqueous solution were concentrated by ethanol precipitation as follows:

1. Sodium acetate (3M), to a final concentration of 0.3M was added, or 7M ammonium acetate to a final concentration 0.7M was added, and the solutions mixed.

2. 2½ vols. ice cold (-20° c) ethanol were added and mixed well. The tubes were left on ice for 20 min.

3. Tubes were spun in a microfuge for half an hour or for larger tubes, these were spun at 10,000 rpm for 30 min. A DNA pellet formed which was washed in 70% (v/v) aqueous ethanol (-20° c), respun for 5 min. and ethanol removed using a drawn - out pipette attached to a vacuum line.

4. The pellet was dried briefly under vacuum and resuspended gently in TE pH 7.5 to a final concentration of 500µg / ml.

2:3:7. **Storage of DNA.**

All DNA, whether single - stranded or RF M13, or plasmid DNA was stored frozen at -20° c.

SECTION 2:4. GEL ELECTROPHORESIS OF NUCLEIC ACIDS.

2:4:1. **High resolution agarose gels.**

When high resolution of DNA fragments was required, agarose gels were made and run in 1X TAE buffer (10X TAE is 48.44g Tris-base, 3.72g EDTA per litre, pH 8.2 with acetic acid) as described by Maniatis et al., (1982). The agarose concentration used (0.6 - 1%), was dependent on the fragment size to be resolved. Agarose medium EEO, type II was used except when the gel was a preparative one in which case low melting point agarose was used. The appropriate weight of agarose was dissolved in 1X TAE using a microwave oven at low power for 5 min. The gel was cooled to 60°c before pouring. Leicester Biocentre gel tanks were used, 10cm or 15cm gels were poured using either 200 or 350ml of 1X TAE agarose. The type of comb used was dependent on the sample size and the number of samples to be run on the gel. The gel was left to set for at least 1 hour then placed in the gel tank and covered in either 1.5 or 2.5 litres of 1X TAE buffer depending on the gel size. The samples were mixed with 5X sample

loading buffer (50% v/v glycerol, 50mM EDTA, 0.1% w/v bromophenol blue) and loaded into the wells. 10cm gels were run at 35V for 16 hours and 15cm gels were run at 60V for 18 hours. The gels were stained in 0.1mg / ml EtBr for 30 min. and were visualised on a UV transilluminator and photographed with a polaroid instant camera using either Polaroid 665 or 667 film

2:4:2. **Rapid analysis agarose gels.**

For the rapid analysis of restriction enzyme digestion products or for DNA quantification, a mini - gel tank (BRL), was used. A volume of 50ml of 0.8% (w/v) agarose in 1x TBE (10x TBE is; 108g Tris base, 55g boric acid, 9.5g EDTA per litre of sterile distilled water.) (Maniatis *et* al., 1982), was prepared by melting the agarose in 1x TBE using a microwave oven. The molten gel was cooled to 60° c and EtBr added to 0.1mg/ml. The gel was poured into the gel tank containing an 8 - well comb and left to set for 30 min. The comb was removed and the gel covered in 1x TBE buffer. To each sample, 5x loading buffer was added, and then loaded into the wells. The gel was run for 30 min. at 70mA and bands visualised and photographed as described above, (Section 2:4:1.).

2:13. **Denaturing formaldehyde agarose gels.**

For the resolution of SP6 or T7 transcription products, formaldehyde - agarose gels were made and run in 1x MOPS buffer, (10x MOPS is 0.2M MOPS (sigma number M-1254), 0.05M sodium acetate, 0.01M EDTA pH 7 with NaOH). Agarose medium EEO, type II, was used at a concentration of 1.5%. A 100ml gel was made by dissolving 1.5g agarose in 75ml sterile distilled water and 10ml 10x MOPS buffer in a microwave oven. When the agarose had cooled to 60° c, 15ml of filtered formaldehyde was added, mixed and the gel poured into a standard 15cm flat bed gel plate with a tape - surround to contain the molten agarose until it had set and a teflon gel comb inserted. The gel was left to set for 1 hour in a fume hood then placed in the gel tank and covered in 1x MOPS buffer. To prepare the samples for electrophoresis, they were denatured in a denaturing solution made up as follows; 100µl of 10x MOPS buffer and 150µl of filtered formaldehyde were added to a 500µl aliquot of deionized formamide. Formamide was deionized by stirring it with BDH " Amberlite " monobed resin MB-3 till it was pH 7. The resin was removed by vacuum filtration through Whatman No. 1 filter paper. A sample of 1 - 5µl of the transcription reaction (see section 2:10.), was added to 15µl of the denaturing solution in an eppendorf and heated at 60° c for 5 min. The tubes were cooled on ice and 2µl of loading mix added (50% glycerol, 0.2% bromo phenol blue), wells were flushed out to remove formaldehyde and the samples loaded. The gel was run at 40mA for 2 - 3½ hours, dried down at 60 - 80°c

under vacuum onto Whatman 3MM, and exposed to Fuji RX X- ray film at -80° c for 4 - 12 hours.

2:4:4. Formamide gels.

For the resolution of ribosomal RNA species, formamide gels were made and run in 0.1% (v/v) TBP,(1x TBP in 36mM Tris, 30mM $NaH_2PO_4(2H_2O)$, 2mM EDTA pH 8.0). A 1.2% (w/v) Agarose formamide gel was made in 50% (v/v) formamide (deionised), 0.1% (v/v) TBP. A carefully cleaned comb was inserted, and when the agarose had cooled to 60°c, the gel was placed in the gel tank and running buffer added level to but not over the gel. 20µl of RNA sample buffer, (60%, v/v formamide (deionised), 0.1% TBP), was placed in each well, then the samples loaded. The gel was electrophoresed at 20mA constant current for 2 - 3 hours. Following electrophoresis the gel was stained with ethidium bromide for 15 minutes then photographed.

2:4:5. Sequencing (8% and 16%) polyacrylamide gels.

2:4:5:1 16% Slab gels.

Two glass plates, 20 x 40cm, one of which was notched, were cleaned thoroughly with detergent and hot water and then ethanol. After drying with tissues, spacers made from 0.2mm plastic card (BRL), were put down each side of the notched plate and a spacer put in the

bottom and assembled under the manufacturers instructions. The plates were clipped together using foldback clips. For a 50ml gel (40 x 20 x 0.02 cm), the following was prepared in a glass measuring cylinder:

21g Urea
7.5ml acrylamide stock solution
5.0ml 10 x TBE buffer
made up to 50ml with sterile distilled water

Acrylamide stock solution is;

38g Acrylamide
2g bis - acrylamide
made up to 100ml with sterile distilled water. 5g of Amberlite monobed resin MB1 was added and stirred for 30 min., then filtered through Whatman No.1 to remove resin.

10 x TBE buffer is;

108g Tris base
55g boric acid
9.3g EDTA made up to 1 litre with sterile distilled water.

The glass cylinder was warmed to 37° c to dissolve the Urea, cooled on ice for 15 - 30 minutes, then 300µl of 10% (w/v) fresh APS and 50µl of TEMED were added and mixed. Using a 25ml pipette, the gel

was carefully poured by tilting the plates at an angle of 30° from the horizontal and sloped slightly so that the acrylamide solution could be poured down the side of the plates. In doing this, the solution filled slowly from the bottom, without forming bubbles. A sharks - tooth comb was inserted and fold back clips used to hold it in place. The gel was left to set for at least one hour, covering the open end with cling - film to reduce dehydration.

The fold - back clips were removed and the bottom spacer removed. The gel was clamped onto the gel apparatus and the buffer tanks filled immediately with freshly prepared 1 x TBE buffer. The wells were thoroughly washed out using a 25ml syringe fitted with a needle, leaving the sharkstooth comb in place. Finally, any air bubbles along the lower end of the gel were washed out using a syringe fitted with a bent needle to ensure even current flowing through the gel.

2:4:5:2. 6% Buffer gradient gels.

Acrylamide stock solution; 40% 19:1 Acrylamide: bis

0.5 x TBE gel mix;	6% (19:1) acrylamide, 0.5 x TBE, 46% (w/v) urea.
2.5 x TBE gel mix;	6% (19:1) acrylamide, 2.5 x TBE, 46% (w/v) urea, 5% (w/v) sucrose, 50µg/ml bromo phenol blue.

Electrophoresis through a supporting medium is termed zone electrophoresis and results in the separation of charged species in different zones. The interval between zones of single - stranded DNA species differing in size by one nucleotide diminishes in a roughly logarithmic manner as the chain length of the DNA increases. Consequently, the lower half of the sequencing gel provides little information compared to the upper half. Biggin and colleagues, (1983) developed buffer gradient gels in which the ionic strength of the electrolyte increases down the length of the gel, resulting in compression of the zones at the lower end of the gel, providing a greater amount of sequence information.

The method used in this study was modified as follows:

10ul of TEMED and 120ul of fresh APS were added to 20ml of 0.5 x TBE gel mix. 5ul TEMED and 60ul APS were added to 2.5 x TBE gel mix. The 0.5 x TBE gel mix was taken up into a 25ml pipette followed by the 2.5 x TBE gel mix. A gradient was formed by the introduction of 5 bubbles of air at the bottom of the pipette. This gradient was poured between glass plates as set up and poured above. A sharkstooth comb was inserted into the top of the gel, covered with clingfilm and allowed to polymerise at room temperature.

The buffer reservoirs were filled with freshly prepared 1 x TBE and electrophoresis was carried out at a constant 30 V/cm. Running the bromo phenol blue to the bottom of the gel allowed sequence

determination from about base 150 to about base 450, roughly equivalent to electrophoresis for three times this length of time in a non - gradient gel.

SECTION 2:5 NUCLEIC ACID RESTRICTION AND MODIFICATION REACTIONS.

2:5:1 Restriction endonuclease digestion of DNA.

Restriction reactions were carried out according to the manufacturers instructions with the following modifications;

Plasmid digestions were performed in reaction volumes of between 20 and 100µl, with a DNA concentration below 100ng/µl with 1 - 5 enzyme units per microgram. Reaction times were between 1 - 2 hours at 37° c except in the case of Sma 1. when reactions were carried out at 30° c. In the instances where DNA would not cut to completion under these conditions, Spermidine was added to a final concentration of 4mM.

Reactions were stopped either by the addition of gel loading buffer in the case of samples to be analysed by agarose gel electrophoresis, or by phenol / chloroform extraction for samples that were to be used for further enzymatic manipulations.

Where appropriate, samples were concentrated following digestion by ethanol precipitation.

2:5:2. Dephosphorylation of vector DNA using calf intestinal phosphatase.

Restricted plasmid DNA (1µg), was phenol / chloroform extracted and ethanol precipitated. DNA was resuspended in 17µl of sterile distilled water.

Removal of terminal 5' - phosphate groups was achieved as outlined in Maniatis et al., (1982); 2µl of 10 x CIP buffer (500mM Tris HCl, pH 9, 10mM Spermidine, 10mM $MgCl_2$, 1mM $ZnCl_2$), was added with 1µl of 1unit / µl CIP. For 3' - recessed ends the reaction mixture was incubated at 37° c for 30 min. followed by the addition of a further unit of CIP and a further 30 min. incubation. In the case of 5' - recessed, or blunt ends, the reaction was carried out for 15 min. at 37° c followed by 15 min. at 56° c. The reaction was terminated by the addition of nitrilo - tri acetic acid to 10mM and heating to 70° c for 10 min. to denature the CIP. The vector was then precipitated and washed (see 2:3:6.), then resuspended in 10µl of sterile distilled water. Dephosphorylated vector DNA was stored at 4° c.

2:5:3 **Ligation of DNA with T4 DNA ligase.**

Ligation reactions were carried out in 20ul reaction volumes, containing 50 - 100ng of dephosphorylated vector and 50 - 100ng of the insert fragment. One unit of T4 DNA ligase was used per reaction with 2µl of fresh 10 x ligation buffer (700mM Tris HCl pH 7.5, 70mM $MgCl_2$), 2µl 100mM DTT, 2µl 10mM rATP, with the remaining volume made up with vector and insert DNA and sterile distilled water. The reactions were incubated at room temperature for 2 - 15 hours and 2µl used per bacterial transformation.

2.5.4 **End - filling reaction.**

T4 Polymerase 1 was used to end - fill 5' overhangs. In a 20µl volume, 1µg of DNA was mixed with 2µl of 10 x T4 Polymerase buffer (670 mM Tris HCl, pH 8.8, 66mM $MgCl_2$, 166mM $(NH_4)_2SO_4$, 67µM EDTA), 2µl, 2% (w/v) BSA, 1µl, 1:100 2-Mercaptoethanol, 1µl 5mM dNTP's and 1 - 2 units of T4 Polymerase. The volume was made up to 20µl with sterile distilled water and the reaction carried out at 37° c for 10 min., then at 15° c for 30 min. The reaction was stopped by phenol / chloroform extraction and the DNA purified by ethanol precipitation.

2.5.5 **Nibble - back reaction.**

T4 Polymerase 1 was again used, to nibble back 3' overhangs produced by Kpn 1, Pst 1 or Sph 1 digestion. 1µg of DNA was mixed with 2µl of 10 x T4 Polymerase buffer (see 2.5.4.), 2µl of 2% (w/v) BSA, 1µl 1:100 2 - mercaptoethanol and 1 - 2 units of T4 DNA polymerase. The volume was made up to 20µl with sterile distilled water. The reaction was carried out for 5 min. at 37° c. To completely blunt - end the DNA, after 5 min., any overhangs not completely removed were filled in using 1µl 5mM dNTP's for 5 min. at 37° c followed by 30 min. at 15° c. The procedure was then the same as in section 2.5.4.

SECTION 2:6 ISOLATION OF DNA FOLLOWING ELECTROPHORESIS.

2:6:1 Elution of DNA from agarose gels.

2:6:1:1 Elution onto filter paper.

(Dretzen et al., 1981).

A 1% agarose gel was run as in section 2:4:1 in the presence of ethidium bromide. Under U.V. illumination from a hand - held source a slit was cut in the gel just in front of the band, using a razor. A piece of Whatman No. 1 was cut to size to fit the slit and backed with dialysis membrane (previously boiled in 20mM EDTA for several minutes).

The edges of the slit were pulled apart and the paper and membrane inserted, so that the paper was towards the DNA. The band was run into the paper and then both this and the membrane were placed in the bottom of a 0.6ml eppendorf tube with a hole pierced in the bottom, which was then placed in a 1.5 ml eppendorf. The tube was spun at top speed in an eppendorf centrifuge for 2 min then 100µl of 10mM Tris HCl pH 7.5 was added to the paper, left a short time then spun again. The small tube was discarded. The 1.5 ml eppendorf was spun for 3 - 5 min. to pellet down any agarose and paper fibre, the supernatant transferred into a fresh tube, phenol / chloroform extracted and ethanol precipitated.

2:6:1:2 Low melting point agarose.

A 1.2% agarose gel was made as in section 2:4:1, however, low melting point agarose was used. Under hand - held U.V. illumination the desired band was cut out as a slice of volume 50µl. Three to five volumes of TE (10 mM Tris HCl pH 7.5, 1 mM EDTA,), were added and

the agarose dissolved by heating to 70° c for 10 min. until all of the agarose had dissolved. An equal volume of phenol (no chloroform), was heated to 70° c and used to phenol extract the molten agarose thoroughly. This was repeated several times till there was no material at the interface. Ethanol precipitation was then as standard.

2:6:2 **Elution of DNA from polyacrylamide gels.**

(Maxam and Gilbert, 1980)

This method was used exclusively for the purification of oligonucleotides from 16% polyacrylamide gels prior to kinasing and site directed mutagenesis.

16% sequencing gels were run as in section 2:4:5:1, and stopped when the bromo phenol blue marker dye had run half the way down the gel. The gel was stained in reservoir buffer with 0.5mg / ml EtBr for 1 min., then, using a U.V. light box a slice corresponding to the correctly - sized oligonucleotide was cut out. The slice was placed in an eppendorf tube and 0.5M Ammonium acetate, 10mM Magnesium acetate 0.1% (w/v) SDS was added as a volume of approximately 0.5ml. The tube was incubated for two hours or overnight at 37° c, phenol / chloroform extracted and ethanol precipitated with 10µg/ml glycogen as a carrier (tRNA was not used as a carrier in this instance since it would interfere with subsequent kinasing).

SECTION 2:7. BACTERIAL TRANSFORMATION.

2:7:1 Preparation of E. coli cells competent for transformation

One colony of DH1 or HB101 E. coli were toothpicked under sterile conditions into 10ml of LB broth and grown up at 37° c, shaking at 200rpm overnight. A volume of 25ml of 37° c LB broth was inoculated with 0.5ml of the overnight culture and grown at 37° c, 200rpm to an A_{660} of 0.48. The cells were harvested at 4° c and 5,000rpm for 5 min in an MSE 8 x 50 rotor, the cells were resuspended gently in 0.5 volumes of ice cold 50mM $CaCl_2$ and incubated on ice for 30 - 40 min. The cells were harvested as above and gently resuspended in 1ml ice cold 50mM $CaCl_2$. The cells were incubated on ice for 1 - 24 hours and used when required.

2:7:2. Transformation of E. coli with Plasmid DNA.

A volume of 100µl of competent cells were used per transformation. DNA, up to a volume of 10µl was added to the cells and these were left on ice for 40 min., then heat shocked at 42° c for 1½ min. Because antibiotic - based selection was used, the cells were incubated in 1ml of fresh LB broth at 37° c for 1 hour to allow expression of the antibiotic resistance gene in transformed cells. The cells were spun down in a microcentrifuge for 2 min., resuspended in 100ul of fresh LB broth and plated out on dried LB antibiotic plates. For plasmid selection the only antibiotic used was ampicillin and this was used at a final concentration of 100µg / ml. Colonies were grown

up at 37° c overnight. Competent cells prepared in this way gave a transformation efficiency of about 1 x 10^7 colonies per microgram of DNA.

2.7.3 **Transformation of E. coli with single - stranded or RF DNA.**

When M13 DNA was used in the transformation of, the heat - shocked cells were added to 3ml of B - Top (10g Bacto tryptone, 5g NaCl, 8g Agar per litre of sterile distilled water), at 42 °c to which had been added, 100µl of a 3 hour culture of the E. coli strain used in the transformation, 25µl of 25mg/ml X - gal and 25 µl of 25mg / ml IPTG. The mixture was then poured onto dried LB agar plates and after the B - Top had set, the plates were inverted and incubated at 37°c overnight. Competent cells prepared in this way gave a transformation efficiency of about 1 x 10^7 plaques per microgram of DNA.

SECTION 2:8. OLIGONUCLEOTIDE SITE - DIRECTED MUTAGENESIS

Oligonucleotides for this work were synthesised and HPLC purified by ICI, (Alderley Edge), Cheshire. Some of the oligonucleotides provided required further purification. Reactions were carried out as described in the Anglian Biotechnology manual and are derived from the methods of Zoller and Smith, (1982).

2:8:1. Construction of template.

An EcoR 1/Hind III fragment containing the full ricin A chain sequence was cloned into M13mp18 (Messing and Vieira, 1982) see figure 8.2.1.

Single - stranded template was prepared exactly as in section 2:3:3.

2:8:2. Sequencing the template with ^{35}S - ATP.

Annealing the primer to the template.

5ul of single - stranded DNA, 1.5ul of 10 x Klenow reaction buffer, 1.0ul of M13 sequencing primer (0.1ug/ul) and 2.5ul of distilled water were mixed in a small eppendorf tube, (10 x Klenow reaction buffer is; 100mM Tris pH 8, 0.5mM $MgCl_2$). The tubes were heated at 95°c for 5 min., mounted in pyrex tubes in a beaker of boiling water, then cooled slowly to room temperature by standing the pyrex tubes containing the eppendorfs in a rack on the bench for up to two hours.

Sequencing reaction,

for each clone to be sequenced, four tubes were labelled G, A, T, C. To each tube was added 2µl of the relevant deoxy/dideoxynucleotide mix, the composition of these mixes is shown below;

TABLE 2:8:1.

Preparation of deoxy/dideoxynucleotide mixes.

---

1. Make up following deoxynucleotide working solutions:

   0.5mM dCTP

   0.5mM dGTP

   0.5mM dTTP

2. Make up deoxynucleotide mixes (A^c, C^c, G^c, T^c,) with the working solutions;

	A^c	C^c	G^c	T^c
0.5mM dCTP	20µl	1µl	20µl	20µl
0.5mM dGTP	20µl	20µl	1µl	20µl
0.5mM dTTP	20µl	20µl	20µl	1µl
1 x TE pH 8.0	20µl	20µl	20µl	20µl

3. Make up dideoxynucleotide working solutions;

   0.10mM ddATP

   0.02mM ddCTP

   0.05mM ddGTP

   0.5mM ddTTP

4. Make up dNTP/ddNTP mixes

   To each dNTP add an equal volume of corresponding ddNTP working solution. This is the dN^c/ddN mix.

The cooled annealed primer and template solution was collected by briefly spinning it to the bottom of the eppendorf. To this was added 2µl of ^{35}S ATP (10µCi/µl), 1µl of fresh 100mM DTT and 1µl of 1µ/µl Klenow fragment diluted fresh in 1 x Klenow reaction buffer.

An aliquot of 3.5µl of this mix was added to the side of each of the tubes containing the 2µl aliquots of deoxy/dideoxynucleotide mixes. Thus, in tube G was 3.5µl of template, primer, label,enzyme & 2µl G°/ddG

"	A	"	"	2µl A°/ddA
	T	"	"	2µl T°/ddT
"	C	"	"	2µl C°/ddC

The tubes were incubated at 30°c for 20 min., in a water bath, at the end of this time, 1µl 0.5mM ATP was added (Chase solution) and the tubes incubated for a further 20 min. at 30°c.

If the samples were to be electrophoresed immediately, 4µl of formamide dye mix was added (mix is; 100µl deionized formamide, 0.1g Xylene cyanol FF, (sigma), 0.1g bromo phenol blue, (sigma), 2ml 0.5M EDTA pH 8.0), to the side of the tube and spun briefly to stop the reactions. Tubes were heated at 100°c for 5 min. with the lid of the tube open to drive off excess water and thereby leave the DNA in a fully denatured state. Tubes were left to cool on the bench and 5µl of each loaded on a prerun 8% buffer gradient gel as described in section 2:4:5:2.

2:8:3. Kinasing the mutagenic oligonucleotide.

Enough oligonucleotide was kinased for several experiments by the following method: The reaction mix contained 10µl of 10pmol/µl oligonucleotide primer, 2µl 10 x Kinase buffer (500mM

Tris HCl pH8.0, 100mM $MgCl_2$), 1µl 100mM DTT, 2µl 10mM rATP made up to 20µl with sterile distilled water. Five units of polynucleotide kinase (Amersham), were added and the reaction incubated for 30 min. at 37°c, then 5 min. at 65°c and frozen at -20°c for storage.

2:8:4. **Annealing the mutagenic oligonucleotide with the template.**

The primer and template were annealed in the smallest eppendorf tubes available to minimise problems of evaporation during high - temperature incubation. The annealing reaction contained 2µl kinased primer (5pmol/ul), 500ng template, 1ul 10 x TM buffer, (100mM Tris HCl pH 8.0, 100mM $MgCl_2$) and made up to 10µl with sterile distilled water. Annealing was carried out at 100°c for 30min. in a small beaker of water and cooled to room temperature over a period of 4 hours to allow the most efficient annealing possible.

2:8:5. **Extension / ligation reaction and E. coli transformation.**

To the annealing mix, 1µl 10 x TM buffer, 1µl 5mM rATP, 1µl 5mM dNTP's 1µl 100mM DTT and 4µl sterile distilled water were added. Ten units of T4 DNA ligase and 2 units of freshly diluted Klenow were added and the reaction incubated at 15°c for 12 - 20 hours. The reaction was diluted to 100µl with sterile distilled water and frozen at -20°c for storage. Aliquots of the mix were then used to transform competent 71:18 MutL E. coli cells as in section 2:7:3.

2:8:6. **Griding - out plaques.**

In preparation forscreening, individual clear plaques were toothpicked onto duplicate LB agar plates in the form of a symmetrical 100 - dot grid. Plaques were grown for 10 hours at 37°c as infected colonies.

2:8:7. **^{32}P - kinasing the mutagenic oligonucleotide.**

Three microlitres of 10 x Kinase buffer, 1µl 100mM DTT, 20pmol oligonucleotide, 3µl (30uCi), [^{32}P] ATP, 2.5 units of T4 polynucleotide kinase were made up to 30µl with sterile distilled water and incubated for 30 min. at 37°c. The reaction was then added to 3ml of 6 x SSC, filtered through a 0.2 micron pore filter into a petri dish and stored frozen at -20°c until required.

2:8:8. **Colony screening.**

Colonies grown up as a grid were transferred to a nitrocellulose filter by pressing the filter onto the agar surface. The filter was then placed, colony side up successively on to 3MM sheets soaked in the following series of solutions;

1. 1 x 3 min. 0.5M NaOH (Lysis)
2. dry 3MM
3. 1 x 3 min. 0.5M NaOH (Lysis)
4. dry 3MM
5. 1 x 1 min. 1M Tris HCl pH 7.4 (Neutralisation)

6. dry 3MM

7. 1 x 1 min. 1M Tris HCl pH 7.4 (Neutralisation)

8. 1 x 5 min. 0.5M Tris HCL pH 7.4,

1.5M NaCl (Fixing)

Once fixed in high salt the filters were air dried, then baked for 1 hour at 80°c in a vacuum oven.

The filters were prewetted for 5 min. in 6 x SSC, then prehybridised in 5ml of 10 x Denhardts, 6 x SSC, 0.2% (w/v) SDS, at 67°c. The filters were rinsed several times in 6 x SSC, then placed colony side down in the thawed probe for 1 hour. For oligonucleotides longer than 20mer, hybridisation to filters was carried out at 67°c for 30 min. then cooled to room temperature for a further 30 min. The filters were dried, washed three times in 6 x SSC at room temperature, monitored, dried on 3MM, wrapped in cling film and autoradiographed at -20°c for 10 min.

Subsequent washing temperatures depended on the base composition of the annealing oligonucleotide probe according to the guidelines laid down by Wallace. (Wallace et al., 1980). Filters were washed in 6 x SSC at 5°c below the dissociation temperature of the oligonucleotide probe (Td), and also at the Td (perfect diasociation temperature), for 3 min. monitoring the presence of hybridising colonies by autoradiography as before. Colonies which gave a strong signal at the Td were picked from the duplicate master plate and introduction of the desired mutation confirmed by sequencing. Single - stranded template was made as in section 2:3:3 and sequenced as in section 2:8:2. RF preps were made by transforming 71:18 E. coli with confirmed mutant single- stranded template as in section 2:7:3 and2:3:4. Mutant ricin A chain sequence was excised using Bam Hi as in section

2:5:1, and ligated, as in section 2:5:3 into the following, specially designed vector, pSP64 Bam. (see chapter 8).

2:8:9. Construction of a vector for the analysis of mutants.

The parental plasmid used in this work was pSP64 XBm and was a gift from Dr. A. Colman, Dept. Biochemistry, University of Birmingham. Plasmid pSP64 XBm was cut with Pvu II and, 12mer Bam Hllinkers (Amersham), were ligated in. This manipulation generated a vector known as pSP64 Bam which has the following characteristics;

1. An SP6 promoter.
2. upstream of this is an ATG codon in a very attractive translation initiation environment, preceeded by the 5' untranslated region of a highly expressed eukaryotic gene (Xenopus B Globin).

Ricin A chain mutant and wild type sequences can be conveniently cloned into the Bam HI site of pSP64 Bam in such a way that the ATG codon provided by the vector acts as a translation start codon for the cloned sequences. See figure 8.2.1.

SECTION 2:9. EXONUCLEASE III DELETION ANALYSIS.

(Henikoff, 1984.)

2:9:1. Exonuclease III reaction.

Ten micrograms of plasmid, pGEM 1A (see figure 3.2.1.)was digested with the enzymes Sst 1 and Xba 1. phenol /

chloroform extracted, ethanol precipitated and dissolved in 100µl of TE (10mM Tris HCl pH 8, 0.1mM EDTA,). This gave enough material for two reactions. Four microlitres of TE as above, 6µl Exonuclease III buffer (500mM Tris HCl pH 8, 50mM $MgCl_2$, 100mM DTT) and 50µl of DNA were mixed and equilibrated at 25°c for 5 min. One hundred units of Exonuclease III were added, mixed rapidly and 10µl removed as quickly as possible into 10µl of 10mM Tris HCl pH 8, 10mM EDTA and kept on ice. Samples were taken at further intervals of 0.5 min. and diluted in equal volumes of TE as before. When all of the samples had been removed, they were heated at 65°c for 5 min. To each 100µl of an S1 nuclease mix was added. (140µl S1 buffer (300mM sodium acetate, pH 4.6, 40mM $ZnSO_4$, 2.5M NaCl), 1.26ml H_2O, 20µl 2mg/ml tRNA and 35 units of S1 nuclease.). Samples were incubated at 37°c for 20 min., phenol / chloroform extracted and ethanol precipitated and taken up in 10µl of 10mM Tris HCl pH 8, 0.1mM EDTA. DNA was then cut with Pst_1. run on a low melting point agarose gel, deleted ricin A chain fragments eluted, phenol extracted and ethanol precipitated.

2.9.2. **Construction of a vector for cloning the deleted sequences.**

The plasmid pSP64 XBm, as used in section 2.8.9., was cut with the enzyme Nco_1. then end - filled as described in section 2.5.4. This created a linearised plasmid with a blunt ATG translation start codon. The linearised DNA was then cut with the enzyme Pst_1 which cuts once in the polylinker region. (see figure 9.2.1.). Into this vector ricin A chain encoding 5' - terminally deleted sequences with a blunt 5' end and a Pst_1 3' end can be conveniently cloned. Subsequent transcription is from the SP6 promoter and translation in

vitro is initiated at the ATG codon provided by the vector. In theory, 33% of the inserts should be in - frame with the ATG codon.

2:9:3. Plasmid sequencing deletion mutants using an SP6 promoter - specific primer.

(Murphy and Kavanagh, 1988)

2:9:3:1 Denaturation of plasmid DNA.

Twenty microlitres of DNA solution made as in section 2:3:2 in 10mM Tris HCl pH 8, 0.1mM EDTA, containing 5µg of DNA was denatured by the addition of 1µl of 5M NaOH and incubation for 5 min. at room temperature.

2:9:3:2. Spin dialysis.

This method rapidly purifies DNA using sepharose CL - 6B (made as a 60%,w/v, slurry (60ml slurry, 40 ml, 10mM Tris HCl pH 8, 0.1mM EDTA), and autoclaved.

The base of a small (0.8ml), eppendorf was punctured using just the tip of a 23 guage x 1.25 syringe needle. The butt of the small tube was filled to a depth of 2mm with glass beads (Ballotini No. 1, acid washed and stored in 10mM Tris pH 8, 0.1mM EDTA. The tube was filled to the rim with Sepharose CL - 6B. The base of a large , (1.5ml) eppendorf was punctured similarly, the small tube placed inside the large and both placed inside a 5ml plastic tube. The tube was spun in a bench centrifuge at 3,000rpm for 10 min. precisely. The column was thus compacted and excess liquid was found in the 5ml tube. The punctured large eppendorf was replaced by an un - punctured tube and placed back in the 5ml tube. The denatured DNA, with 2µl of bromo

phenol blue solution was loaded and spun for exactly the same length of time and speed as earlier. The NaOH and bromo phenol blue remained on the Sepharose CL - 6B and 20µl of liquid containing DNA was recovered from the large eppendorf.

2:9:3:3 Annealing.

Eight microlitres of denatured DNA was put into a fresh 0.8ml eppendorf tube (the rest frozen at -20°c) and 1µl of TM, (100mM Tris HCl pH 8.0, 100mM $MgCl_2$) added. One and a half microlitres of SP6 primer was added, (10ug/ml or $A_{260} = 5 \times 10^{-3}$ units per millilitre). Annealing was at 42°c for 15 min. in a waterbath and at the end of this time the tubes were stored on ice.

2:9:3:4. Sequencing.

Two microlitres of the deoxy / dideoxynucleotide mixes (composition of the mixes is shown below in table 2:9:1.),A, C, G, T, were added into separate, clearly labelled tubes.

Table 2:9:1

composition of deoxy/dideoxy nucleotide mixes in plasmid sequencing

	A	C	G	T
0.5mM dCTP	500µl	25µl	500µl	500µl
0.5mM dGTP	500µl	500µl	25µl	500µl
0.5mM dTTP	500µl	500µl	500µl	25µl
10mM ddATP	1µl	-	-	-
10mM ddCTP	-	8µl	-	-
10mM ddGTP	-	-	16µl	-
10mM ddTTP	-	-	-	50µl
$T_{10}E_{0.1}$ buffer	500µl	1000µl	1000µl	1000µl

The " Klenow mix " was made up as follows:

For 10 clones; 3µl TM

62µl H_2O

15µl ^{35}S dATP (10µCi / ml)

5µl Klenow (5units / µl)

Eight microlitres of the klenow mix was added to the annealing mixture and 4.5µl of this was pipetted onto the wall of each dNTP/ddNTP tube. The reaction was started by briefly spinning the tube and was carried out at 42°c for 10 mins. in a water bath. After 8 min., 2µl of chase mix (0.5mM dNTP mix), was added to the wall of each tube and at 10 min. the chase reaction started by spinning and carried out at 42°c for 5 min. Four microlitres of formamide dye mix was added as in

section 2:8:2, the reaction tube boiled for 2 min. and the reaction loaded on an 8% buffer gradient sequencing gel as in section 2:4:5:2.

SECTION 2:10. SP6 / T7 in vitro TRANSCRIPTION REACTIONS.

(Krieg and Melton, 1984)

The method used in this study was that used in the lab. of D. Mayer, EMBL, Heidelberg, FRG. (personal communication through J. Gould, this lab.)

2:10:1. Preparation of DNA for transcription.

Ten micrograms of DNA was linearised with a restriction enzyme in the polylinker region at the 3' end of the inserted gene, cloned in an SP6 / T7 vector, distal to the polymerase promoter to be used in the transcription reaction. Complete linearisation was checked by running an aliquot of the phenol / chloroform extracted, ethanol precipitated reaction on a 1% agarose mini - gel as in section 2:3:2. DNA was routinely resuspended in 10µl of sterile distilled water to give a final concentration of 1µg / µl; enough for five transcription reactions.

2:10:2. **The RNA synthesis reaction.**

A premix was made up as outlined below:

1. 10 x Transcription salts

		final conc^{n}.
Spermidine	5mg	20mM
1M HEPES pH7.5	400µl	400mM
1M magnesium acetate	60µl	60mM
sterile distilled water	540µl	

2. Stock solution

	For 6ml of mix	final conc^{n}.
10 x Trans^{n}. salts	1000µl	1 x
10mg/ml BSA	100µl	100µg/ml
500mM DTT	100µl	10mM
50mM rUTP, ATP, CTP,	100µl	0.5mM
5mM rGTP	200µl	0.1mM
sterile distilled water	4.5ml	
	6ml	

The above was filtered through a 0.222 micron pore filter, aliquoted into 65µl aliquots, snap - frozen in liquid nitrogen and stored for up to 12 months at -80°c.

A x 1 reaction was set up which can be multiplied up to allow for multiple transcriptions. The reaction was set up as follows;

Premix	12µl
Human placental RNAasin	0.5µl
Sterile distilled water	2.5µl
M7G(5')ppp(5')G (cap)5mM	1.0µl
	10µl

For each reaction 10µl of the above mix was added to 2µl DNA (2µg), and 2µl (10units) of the relevant polymerase was added. The reaction was carried out at 40°c for 30 min., after this time 1µl 8mM rGTP was added and the reaction continued for a further 30 min. If RNA was not to be used immediately, at the end of the reaction, the reaction tube was dropped into liquid nitrogen and stored at -80°c.

2:10:3. **Quantification of the transcription reaction.**

Ten micro curies of ^{3}H UTP was added to each reaction by drying down 10µl (10µCi) from an ethanol suspension and completing the reaction in this tube. Processing was as follows;

2µl of the reaction mix was spotted onto Whatman No. 1 paper discs and air dried. The 1cm discs were washed twice in 10% TCA, 20mM $Na_2P_2O_4$ for five minutes each, twice in ethanol and once in ether, then air dried. Discs were counted 4ml Beckman EP scintillation fluid on an LKB 1212 scintillation counter. Typical quantification calculations are described in section 3:2

Labelled transcripts were visualised as described in section 2:4:3.

SECTION 2:11. CELL - FREE TRANSLATION SYSTEMS

In each translation system used, RNA was added directly as an aliquot of the transcription reaction which had been stored frozen at -80°c. Initially, thorough phenol / chloroform extraction, followed by ethanol precipitation was recommended to purify the transcripts. However, phenol extracted versus non - extracted transcripts were compared for their activity in wheatgerm and rabbit reticulocyte lysates and no difference was observed in their activity.

2:11:1. The wheatgerm lysate reaction.

The basic wheatgerm cell - free lysate system used was initially described by Roberts and Paterson, (1973). Since then, several improvements have been made, (Marcus and Dudock, 1974, Atkins et al., 1975) and the system used in this work was a refinement of the procedure described by Anderson et al., (1983).

Wheatgerm lysate was prepared according to the method of Anderson et al., (1983) and was a gift from Dr. C Robinson, University of Warwick.

An energy - generating system was made up as in table 2:11:1, with the omission of magnesium acetate, which, after optimisation was found to be highly inhibitory even at the levels recommended. Table 2:11:2 shows the concentrations of 19 amino acids used in the energy generating system (after Anderson et al., 1983,).

The components for a wheatgerm lysate were all defrosted from -80°c., just prior to use and kept on ice for as short a period of time

as possible. Samples of wheatgerm lysate were spun for 4 min. in a microfuge at 4°c. as soon as defrosted, prior to use.

Table 2:11:1.

Composition of the wheatgerm lysate energy generating system

		concentration		
		lysate	energy mix	total
*1M HEPES pH 7.6	350µl	6	14	20mM
+0.1M ATP	250µl	-	1	1mM
0.4M creatine phosphate	500µl	-	8	8mM
10mg/ml CP kinase	100µl	-	40	40µg/ml
1.5mg/ml Spermine pH7	500µl	-	30	30µg/ml
0.1M DTT	425µl	0.3	1.7	2mM
#2mM GTP + Mg^{++}	250µl	-	20	20µM
5mM each 19 amino acids	125µl	-	25	25µM
$1M potassium acetate	2100µl	36	84	120mM
magnesium acetate	-	1.5	-	1.5mM

The energy mix was made up and stored at -80°c in 20µl aliquots and defrosted once only for use.

* - 1.0M HEPES adjusted to pH 7.6 with 5M KOH

\+ - 1.0M Potassium acetate adjusted to pH 7 with acetic acid

\# - 0.1M ATP " " with KOH

$ - GTP + magnesium acetate, each 40mM, adjusted to pH 7.

Table 2:112.

composition of the 5mM 19 amino acid stock mix

Acid	0.1M (mg / ml)
L - Alanine	8.9
L - Arginine HCl	21.1
L - Asparagine H_2O	15.0 suspension
L - Aspartic acid	13.3 suspension
L - Cysteine	12.1
L - Glutamic acid	14.7 suspension
L - Glutamine	14.6
Glycine	7.5
L - Histidine - HCl	21.0
L - Isoleucine	13.1
L - Leucine	13.1
L - Lysine - HCl (hydrate)	18.3
L - Phenylalanine	16.5
L - Proline	11.5
L - Serine	10.5
L - Tryptophan	20.4 suspension
L - Threonine	11.9
L - Tyrosine	18.1 suspension
L - Valine	11.7

The wheatgerm lysate reaction was set up as follows:

wheatgerm lysate	3.75µl
Energy mix	2.35µl
RNA	1.0µl
H_2O	4.4µl
^{35}S - Methionine	1.0µl
	12.5µl

This reaction could be scaled up to give 25, 50, 75, or 100µl volumes as required. The reaction was carried out at 28 - 30°c. for 1 hour in a water bath. At the end of the reaction, 2µl aliquots were processed to determine the level of incorporation of ^{35}S - Methionine into Trichloroacetic acid precipitable protein.

1. A 2µl aliquot was spotted onto a Whatman 3MM.1, 1cm disc, pinned onto a board and left to air dry.

2. Discs were washed twice in 10% ice cold TCA for 5 min. each, then for 15 min. in 5% TCA in a boiling water bath, rinsed with cold water and washed in 100% (v/v) ethanol for 1 min. then pinned on a board and left to air dry.

3. TCA precipitable counts were estimated by immersing the dried squares in 4ml Beckman EP scintillation fluid and counting on an LKB 1212 Minibeta scintillation counter.

The remaining 10.5µl of reaction sample was either stored at 4°c for up to 1 month or run on a 10 or 15% Polyacrylamide - SDS gel. (see section 2:12:3.).

2:11:2. **The rabbit reticulocyte lysate system.**

For some reactions, the lysate used was obtained from Amersham and was used according to their instructions.

2:11:2:1. Preparation of lysate.

For most of the reactions, however, the lysate was prepared in the laboratory using a method based on that of Clemens, (1984). Four rabbits were used to prepare the lysate.

Day 1. Rabbits were injected subcutaneously with 1ml of vitamin B_{12} / Folic acid in 0.15M NaCl and 0.6ml of 2.5% phenyl hydrazine in 0.15M NaCl. Vitamin B_{12} / Folic acid solution were made up as follows:

10mg vitamin B_{12}

100mg Folic acid

in 0.15M NaCl adjusted to pH 7 with KOH and made up to 100ml.

Days 2. - 5. Each rabbit was injected with 0.6ml, 2.5% Phenylhydrazine in 0.15M NaCl, per day.

Day 7. 1ml of Pentathol was injected into the ear of each rabbit to anaesthatise them.

Blood was collected by cardiac puncture into 250ml glass centrifuge pots. All glassware was treated with 1000 usp units / ml Heparin (to prevent blood clotting), prior to use. Blood was stored on ice till all the rabbits had been bled and kept ice cold at all times subsequently. Red blood cells were spun down at 2000rpm, 4°c. for 15 min. in an MSE 6L centrifuge and the serum supernatant removed. Wash buffer was added to the cell pellets to resuspend them and then spun down as before. This was repeated 3 - 4 times until the supernatant was clear.

Wash buffer is; 0.14M KCl

50mM NaCl

5mM $MgCl_2$

After the final spin, the supernatant was removed and the packed cell volume of the pellets estimated. An equal volume of iced water was added to lyse the cells which were spun immediately in a HS 18 centrifuge at 8000rpm, 4°c. for 10 min. The lysate supernatant was dispensed into 1ml volumes and stored in liquid nitrogen.

Preparation of the energy generating system.

An amino acid stock solution was made up so that each was at 2.5mM, giving a final concentration of 100μM in the reaction. The composition of this stock is shown in Table 2:12:1. The amino acids were dissolved in 20ml of sterile distilled water, neutralised with KOH to pH 7.4, made up to 25ml, dispensed into 2ml aliquot and stored at -20°c,

For the energy generating system, a 20 x concentrated solution was prepared as follows;

The 10ml ATP - GTP mixture contained,

	final concentration
120mg di - P - ATP	20mM
24mg Na - GTP	4mM
1ml, 2M Tris HCl pH 7.5	200mM
7ml sterile distilled water	

This was neutralised to pH 7.5 with KOH, made up to 10ml, dispensed into 2ml aliquots and frozen for storage for storage at -20°c. When required, 145.2mg of di Tris Creatine phosphate and 2mg of Creatine phosphokinase were added and refrozen at -20°c.

Table 2:12:1

composition of the 2.5ml 19 amino acid stock mix

Acid	mg in 20ml
L - Alanine	89.1
L - Arginine HCl	210.7
L - Asparagine H_2O	132.1
L - Aspartic acid	133.1
L - Cysteine	121
L - Glutamic acid	147.1
L - Glutamine	146.1
Glycine	75.1
L - Histidine HCl	191.7
L - Isoleucine	131.2
L - Leucine	131.2
L - Lysine HCl (hydrate)	189.7
L - Phenylalanine	165.2
L - Proline	115.1
L - Serine	105.2
L - Tryptophan	204.2
L - Threonine	119.1
L - Tyrosine	181.2
L - Valine	117.2

2:11:2:2. Micrococcal nuclease treatment of the lysate.

When required for use the 1ml aliquots of reticulocyte lysate were treated with Staphylococcal nuclease to remove endogenous messenger RNA as follows;

20µl	Creatine phosphokinase	(5mg/ml)
80µl	Haemin	(1mM)
20µl	$CaCl_2$	(100mM)
30µl	Nuclease	(1mg/ml)

The lysate was incubated for 12.5 min. at 20°c. and 10µl, 0.5M EDTA was added to stop the reaction by chelating out the calcium. Nuclease - treated, message - dependant lysate was stored as 50µl aliquots at -80°c.

2:11:2:3. Rabbit reticulocyte lysate reaction.

	1 x reaction (50µl reaction)
Amino acid mix	2µl
Energy mix	2.5µl
1M Potassium acetate	3.75µl
50mM Magnesium acetate	1µl
Wheatgerm tRNA (1mg/ml)	2µl
^{35}S - Methionine	2.5µl
10mM Tris HCl pH 7.4	10µl

This mix could be multiplied up for as many reactions as is required and added to 2µl transcription mix and 25µl of Nuclease - treated, message - dependant lysate, added last to thoroughly mix the reaction. The reaction was set up on ice and carried out at 30°c. in a water bath. At the end of the reaction (1 hour), 2µl samples were taken for processing to assess ^{35}S - Methionine incorporation into TCA precipitable protein exactly as in the wheatgerm reaction.

2:11:3. **The yeast lysate reaction.**

Several methods exist for the production of yeast lysates, (Tuite *et al.*, 1980, Chanda and Kung, 1983). The method used in this study was that described by Rothblatt and Meyer, (1986), using a yeast S - 100 extract, (Gasior *et al.*, 1979), prepared from the vacuolar protease deficient strain, ABYS 1, (Achstetter *et al.*, 1984).

The lysate used was a gift from D. Meyer, EMBL, Heidelberg, FRG. The lysate was treated with Staphylococcal nuclease, (2µl of 30,000units / ml in 20mM HEPES, pH 7.5), with 1µl of 200mM $CaCl_2$, for 20 min. at room temperature (22°c.) and the reaction stopped by the addition of 2µl of 200mM EGTA pH 7.5. Nuclease - treated, message dependent lysate was stored at -70°c until required. An energy generating supplement was prepared with the following components;

Stock solution	in 4ml	final conc°
19 amino acids (1mM)	1.48ml	0.375mM
1M HEPES KOH pH 7.5	1.48ml	375mM
1M DTT	100µl	25mM
tRNA (lyophilised)	10mg	2.5mg/ml
150mM GTP	33.33µl	1.25mM
500mM ATP	100µl	12.5mM
1.5M creatine phosphate	667µl	250mM
*40mg/ml creatine phosphokinase	100µl	1mg/ml
20 mM HEPES pH 7.5	40µl	
TOTAL	4ml	

* creatine phosphokinase prepared in 50% (v/v) glycerol, 20mM HEPES pH 7.5.

19 amino acid mix prepared from a dilution of that made for wheatgerm.

The mix was filtered through a 0.222 micron pore filter, aliquoted into 25µl lots an stored at −80°c.

For a 1 x reaction mix the following was set up;

Energy generating supplement	2µl
Human placental RNAasin	1µl
^{35}S - Methionine	2µl
3M Potassium acetate	1.25µl
50mM Magnesium acetate	0.75µl
Sterile distilled water	7µl
TOTAL	14µl

This was added to 1ul of the transcription reaction and 10µl of nuclease - treated, message - dependent lysate added to give a final volume of 25µl. Reactions were carried out at 25°c. in a water bath for 1 hour. At the end of this time, assessment of the incorporation of ^{35}S - Methionine into TCA precipitable protein was exactly as in section 2:11:1 for the wheatgerm lysate reaction.

SECTION 2:12 ANALYSIS OF CELL - FREE TRANSLATION PRODUCTS.

2:12:1 Protease digestion of ricin A chain polypeptide synthesised from mutant and wild - type sequence

After one hours translation, a wheatgerm lysate was diluted to 150µl with 10mM Tris HCl pH 7 and divided up into 20µl aliquots. Two proteases were used; Thermolysin and Trypsin, (Boehringer). Thermolysin was made up as a 1mg / ml stock in 10mM $CaCl_2$. Trypsin was made up as a 1mg / ml solution in 10mM Tris HCl pH 7. Both stocks were diluted down to 50µg / ml with sterile distilled

water. Both sets of protease digestion reactions were carried out for 10 min. on ice and set up as follows;

To a 20µl wheatgerm lysate sample, add:

volume of protease	final concentration
12µl	15µg / ml
8µl	10 "
4µl	5 "
1µl	2.5 "
0µl	0 "

After the incubation, an equal volume of 2 x SDS sample buffer (see section 2:12:3:1), was added and samples boiled for 5min. prior to electrophoresis as in section 2:12:3:1.

2:12:2 **Immunoprecipitation of ricin polypeptides from cell - free translation systems.**

The cell - free translation was dispersed in an equal volume of 1% (v/v), NP40 buffer containing 40µg / ml fresh PMSF (20mg / ml stock in ethanol made fresh). Any debris was pelleted by a brief 30 sec spin in a microfuge. The supernatant was mixed with 2µl of preimmune serum and incubated for 15 - 30 min. at room temperature. To this was added 25µl of a protein A sepharose slurry (equilibrated as a 1:1 slurry in 1% NP40 buffer: 1% NP40, 10mM Tris HCl pH 7.5, 150 mM NaCl) and incubated for 30 min. at room temperature with occasional shaking to unsettle the beads. The beads were pelleted by a brief spin in a microfuge for 30 seconds and the supernatant retained. To the supernatant was added 2µl of the appropriate rabbit antibody and incubated overnight at 4°c. on a vertical turntable. A volume of

25 - 30ul of protein A Sepharose slurry was added as before for 30 min. at room temperature with occasional shaking. The beads were collected by centrifugation for 30 seconds and washed three times with 0.2% NP40 buffer, pelleting each time as above. The beads were then washed with 0.2% NP40, 10mM Tris HCl pH 7.5, 0.5M NaCl, pelleted, then washed once with 10mM Tris HCl pH 7.5 and pelleted hard for 2 min. in the eppendorf centrifuge. The supernatant was carefully removed and 2 x SDS sample buffer was added to each, the samples boiled for 3 min (to dissociate Ag-Ab and to dissociate Ag-Ab from the beads). Before loading the samples on a polyacrylamide gel, the beads were pelleted hard and the supernatant removed for loading.

2:12:3. Analysis of proteins by polyacrylamide gel electrophoresis.

2:12:3:1 Electrophoresis under denaturing conditions.

The method of Laemlli, (1970), was followed, using slab gels of dimensions 15cm x 15cm x 0.15cm.

Gels were prepared by mixing together the following:

	Percentage gel (acryl:bis)		
	10:0.13	12.5:0.01	15:0.086
30% (w/v) Acrylamide	20ml	25ml	30ml
2% (w/v) bisacrylamide	4ml	3ml	2.6ml
1M Tris HCl pH 8.8	22.4ml	22.4ml	22.4ml
10% (w/v) SDS	0.6ml	0.6ml	0.6ml
sterile distilled water	12.85ml	8.85ml	4.25ml
TEMED	50µl	50µl	50µl
DTT	4mg	4mg	4mg

Just before casting the gel, 200µl of freshly prepared 10% (w/v) ammonium persulphate (APS), solution was added. The gel was overlaid with butan - 1 - ol until polymerisation was complete. The butan - 1 - ol was washed off with water and a stacking gel containing the following was cast on top of the resolving gel;

30% (w/v) acrylamide: 0.8% (w/v) bisacrylamide	6ml
0.5M Tris HCl pH 6.8	7.5ml
sterile distilled water	15ml
10% (w/v) SDS	300µl
TEMED	10µl

Freshly prepared 10% (w/v) APS (100µl), was added just before casting the gel and a slot former was inserted before polymerisation. After polymerisation, the slot former was removed and the gel mounted in a gel tank. Sample solutions were added to an equal volume of sample buffer and boiled for 5 min. Sample buffer is:

0.5M Tris HCl pH6.8	2.5ml
Glycerol	2ml
10% (w/v) SDS	4ml
2 - mercaptoethanol	1ml
bromo phenol blue	0.1g
sterile distilled water	0.5ml

Suitable volumes of samples were loaded and the gel was run at 12mA constant current for 17 hours in a buffer containing;

Tris base pH 8.3	25mM
glycine	192mM
SDS	0.1% (w/v)

2:12:4. **Staining polyacrylamide gels.**

Proteins were visualised by immersion of polyacrylamide gels in the following solution for 1 hour;

Coomassie brilliant blue R	0.25% (w/v)
Methanol	50% (v/v)
Acetic acid	7% (v/v)

Excess stain was removed by washing the gel in a number of changes of 40% (v/v) methanol, 7% (v/v) acetic acid.

2:12:5 **Fluorography of polyacrylamide gels.**

Fluorography of destained gels was simply by immersing the gel in Amplify, (Amersham), solution for 15 - 30 min. before drying down onto Whatman 3°. 1 paper under vacuum for 1 hour at 60 - 80°c.

2:12:6 **Autoradiography of polyacrylamide gels.**

Autoradiography of dried gels was by exposure to Fuji RX X - Ray film for 1 - 20 hours at -80°c.

CHAPTER 3.

CLONING RICIN cDNA SEQUENCES INTO TRANSCRIPTION VECTORS

SECTION 3:1. INTRODUCTION

3:1:1 **The development of in vitro transcription systems.**

Prior to 1978, progress in understanding the mechanisms controlling the temporal program of eukaryotic gene expression had been hindered by an inability to study the process *in vitro*. The development of cell-free transcription systems that direct accurate initiation of RNA polymerase II and III opened up the way to a detailed biochemical analysis of transcriptional regulation in higher organisms (Wu, 1978; Weil *et al.*, 1979; Roeder, 1979; and Manley *et al.*, 1980). Early *in vitro* RNA synthesis techniques relied on:

1. The use of cloned viral promoters from which RNA polymerases II and III could selectively initiate RNA synthesis. The promoters of choice were the Ad2 late promoter, (Weil *et al.*, 1979 and Manley *et al.*, 1980), and the SV40 early and late promoters, (Rio *et al.*, 1980).

or, 2. The use of dialysed and concentrated whole-cell extracts derived from cultured animal cells, (eg. Manley *et al.*, 1980.),which either contain, or can be supplemented with eukaryotic RNA polymerases II and III.

These *in vitro* transcription systems allowed the synthesis of up to 0.006 pmol/hour "run off"transcripts (Rio *et al.*, 1980) and thus facilitated the biochemical analysis of transcriptional regulation *in vitro*. Furthermore, the synthesis of a desired RNA molecule *in vitro*

circumvents problems associated with isolating rare RNA's in amounts sufficient for detailed biochemical analysis.

Other methods were reported for synthesising RNA's in vitro. These involved the use of E.coli RNA polymerase to transcribe DNA's cloned downstream of a eukaryotic promoter, (Roberts, 1969, Blattner and Dahlberg, 1972, Rosenberg et al., 1975, Roberts et al., 1975, Patterson and Rosenberg, 1979).

However none of these methods were ideally suited for the synthesis of large amounts of a specific RNA molecule because of inefficient RNA synthesis, transcription of both DNA strands, incorrect initiation, premature termination, or a combination of these problems.

In order to synthesise large amounts of any specific RNA and to generate RNA's of a high specific activity, it is necessary to have a transcription system which will initiate and terminate RNA synthesis efficiently at precise points on the DNA template. It is also desirable that it works efficiently in a simple buffer, eliminating the need to prepare and characterise cell extracts.

3:1:1:1. The SP6/T7 system.

The SP6/T7 in vitro transcription system was developed to circumvent the problems associated with the early in vitro transcription systems. Several investigators exploited the specificty of phage RNA polymerases and replicases to produce defined RNA's in vitro.

1. McAllister and colleagues found T7 RNA polymerase would efficiently initiate RNA synthesis at cloned T7 promoters (McAllister et al., 1981).

2. Miele and colleagues found QB replicase could be used to produce large amounts of RNA in vitro by autocatalytic replication of recombinant RNA (Miele et al., 1983).

3. Chamberlin and colleagues deacribed a very attractive system using an unusually specific promoter-RNA polymerase combination found in the Salmonella typhimurium phage SP6 (Miele et al., 1983).

Using the SP6/T7 systems in particular, an efficient in vitro transcription system has been developed by Melton and his colleagues (Melton et al., 1984).

The phage encoded RNA polymerases efficiently initiate transcription only at specific promoters and will transcribe any DNA sequence cloned downstream from these promoters. Transcripts resulting from initiation at other prokaryotic or eukaryotic promoters, and to end transcription of DNA restriction fragments, or transcription of the wrong (normally non-transcribed) DNA strand are rarely, if ever, observed. Moreover, the transcription system consists of a simple salt buffer, DNA template, ribonucleoside triphosphates and SP6 orT7 RNA polymerase, and results in the synthesis of microgram quantities of RNA.

Thus, the SP6/T7 in vitro transcription system represents a simple and efficient method for synthesising pure, single- stranded RNA's from virtually any DNA template. This in vitro transcription system is based on the unusually specific RNA synthesis by the RNA polymerases ofbacteriophages SP6 or T7 which initiate transcription exclusively at an SP6 or T7 promoter respectively. (Butler and

Chamberlin, 1982, Kassavetis et al., 1982). Transcription vectors in which the gene of interest is cloned downstream from the SP6 or T7 promoter in a pUC- based plasmid were developed and described by Melton et al., (1984).

Figure 3.1.1. represents the scheme of events undertaken from cloning a gene of interest into a transcription vector, through to producing microgram quantities of RNA. The transcription vector is linearised with restriction enzymes cutting in the polylinker region producing 5'and 3' termini compatible with the 5' and 3' ends of the fragment to be inserted. Alternatively, the fragment can be blunt- end ligated into the linearised vector at any point in the polylinker region. The orientation of the fragment of interest is confirmed by restriction enzyme digestion. The chimaeric plasmid is linearised at the 3' end of the gene of interest. Incubation of purified, linearised plasmid with rNTP's, a simple buffer and the phage polymerase whose promoter is adjacent to the 5' end of the gene of interest produces "run off" transcripts. DNAase treatment is optional and only essential where labelled transcripts are to be used in hybridisation studies.

The commercial availability of a wide range of SP6/T7 vectors and the availability of purified polymerases within the last five years have had a major impact on many areas of molecular research. Indeed it could be said that since their inception, research in the areas described below have been revolutionised as these techniques have been refined.

1. The *in vitro* synthesis of unprocessed RNA's has greatly facilitated studies on RNA splicing (Green et al., 1983, Krainer et al., 1984) and 3' end formation (Krieg and Melton 1984a).

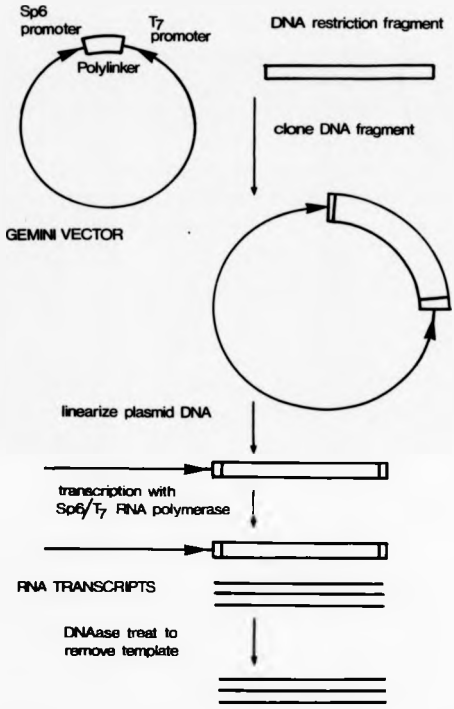


Figure 3·1·1 **THE Sp6/T7 In vitro TRANSCRIPTION SYSTEM**

2. Single-stranded SP6/T7 transcripts synthesised *in vitro* can be labelled to very high specific activities for use as sensitive hybridisation probes (Melton *et al.*, 1984, Cox *et al.*, 1984, Angerer *et al.*, 1981,). These RNA probes are in some cases preferable to conventional nick- translated DNA probes because they are easy to prepare and can increase the sensitivity of the detection method. (Zinn *et al.*, 1983).

3. Microgram quantities of eukaryotic mRNA's can be easily made by this method. Previously, it was difficult to obtain stable and efficient transcription *in vitro*. By the use of the SP6/T7 system, pure wild-type or mutant RNA's can be synthesised and their function tested directly either *in vivo*. after microinjection (Krieg and Melton 1984b, Richardson *et al.*, 1988) or *in vitro* (this work).

4. The analysis of rare or unstable RNA's is now possible, also the analysis of unstable proteins or proteins with short half-lives is possible after transcription and translation of the cloned gene (Glass *et al.*, 1987).

Of importance in their contribution to the impact of this system are the following features of it.

1. The commercial availability of a wide range of high copy number vectors, and of purified polymerases. In particular the development of cloned T7 RNA polymerase has dramatically cut the cost involved.

2. The transcription system works efficiently in a simple buffer, eliminating the need to prepare and characterise cell extracts.

3. Transcription is of one DNA strand at a time, allowing the production of either microgram quantities of sense- strand or the production of anti- sense RNA which can be labelled to a very high specific activity for use in hybridisation studies.

4. This system will initiate and terminate RNA synthesis efficiently at precise positions on the DNA template.

5. Transcription is initiated exclusively at a single promoter.

3:1:2. Commercially available SP6/T7 transcription vectors.

A transcription vector needs a minimum of four functions. These are:

1. An origin of replication, allowing maintainance of the plasmid in the host organism of choice. The most commonly used origin is that derived from the E. coli plasmid pBR322 (Bolivar and Rodriguez, 1974),

2. A selectable marker, usually the B-lactamase gene of pBR322 or pUC, conferring resistance to ampicillin,

3. A specific promoter from which transcription can be initiated,

and 4. Immediately downstream of this, a region designed for cloning DNA fragments.

In addition, insertional inactivation of a marker gene would be an advantage, although it is not absolutely necessary.

Melton and colleagues,developed the first convenient cloning vectors that contained an SP6 promoter immediately upstream from a polylinker region (Melton et al ., 1984.).

Since then, the original vectors have undergone modifications, largely in the polylinker region, allowing the cloning of a wider

variety of DNA fragments. Many of these vectors are now commercially available, as are the phage polymerases.

The vectors employed in this work are described briefly below.

pSP64

Full details of the construction of this plasmid are described in Melton et al.,(1984). Figure 3.1.2a.. shows the main features of this vector and details of the polylinker and SP6 promoter region.

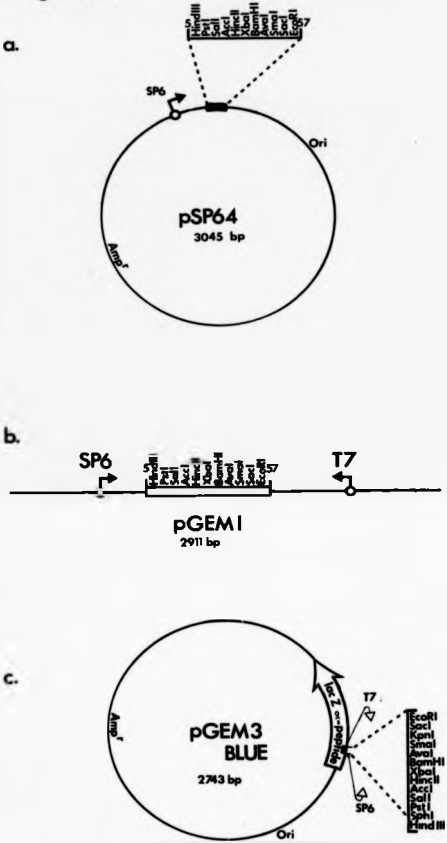
pGEM1 and pGEM2

According to the manufacturers (Promega Biotec, Madison, U.S.A.), the Riboprobe Gemini system "represents the latest in RNA probe technology". The advantage of these vectors is that RNA synthesis can occur from either DNA strand following a single plasmid construction. This is possible since the vectors contain both the SP6 and T7 promoters flanking a polylinker region. Otherwise the vectors are more or less identical to pSP64. Details of the pGEM1 polylinker and promoter region are shown in figure 3.1.2.b. The pGEM2 polylinker region is identical but reversed.

pGEM_BLUE

The pGEM BLUE vector system (Promega Biotec), represents a further refinement in RNA synthesis technology. Essentially, the vectors are the same as above, comprising SP6 and T7 promoters placed either side of a polylinker region, ampicillin selection and an origin of replication. However, in addition, they carry the lac Z - peptide, thus permitting colour selection of recombinant plasmids, (Yanisch-Perron,et al., 1985). Details of this vector are shown in figure 3.1.2.c.

Figure 3.1.2.


a.
SP6
Ori
pSP64
3045 bp
Amp^r
b.
SP6
T7
pGEM1
2911 bp
c.
pGEM3
BLUE
2743 bp
Amp^r
Ori
T7
SP6

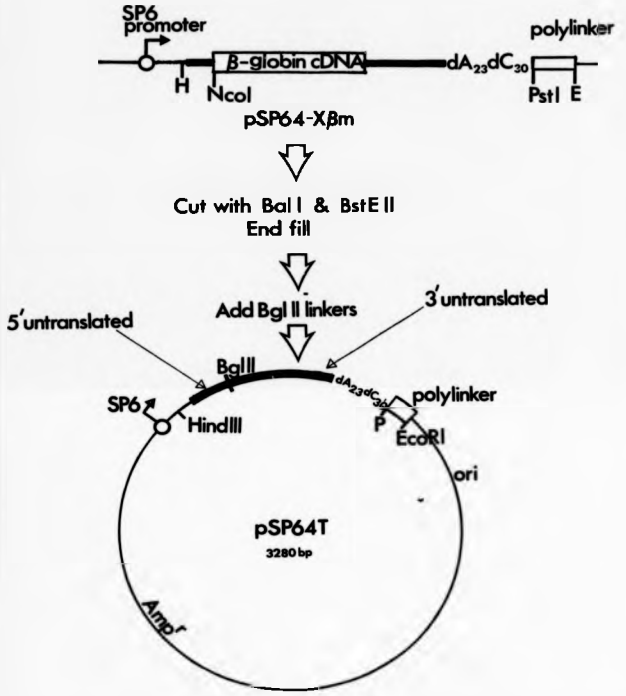
3:1:3. The vectors pSP64-XBm and pSP64T

These two vectors are described in full in Krieg and Melton, (1984), and were a gift from Prof. Alan Colman, University of Birmingham, U.K.

pSP64-XBm contains a Xenopus B-globin cDNA clone inserted into pSP64. B-globin RNA's transcribed from this plasmid contain an extra 12 bases at their 5' end and a stretch of about 30 C residues at the 3'end after the poly (A) tail. This vector was tailored to suit the requirements of the present study as described in chapters 8 and 9. The region from the SP6 promoter to the polylinker region is shown in figure 3.1.3.

pSP64T is an SP6 cloning vector designed to provide 5' and 3' flanking regions from an mRNA which is efficiently translated (B-globin), to any cDNA which contains its own initiating codon. This vector was termed a "translation" SP6 vector by Krieg and Melton since mRNA transcribed from it is in a conformation suitable for translation in vitro or in vivo after microinjection into suitable eukaryotic cells. Its construction is covered in detail in Krieg and Melton, 1984. For my purposes it was considered an excellent vector for the synthesis in vitro or in Xenopus oocytes of ricin A chain from cDNA's cloned into it. However, the only cDNA which I found could be cloned into this vector was proricin as described in section 3.2.3. In spite of numerous attempts, it was impossible to clone a ricin A chain cDNA into this vector. Plasmid pSP64T is illustrated in figure 3.1.3.

Figure 3·1·3.


SP6 promoter
polylinker
β-globin cDNA
dA23dC30
H
NcoI
PstI
E
pSP64-Xβm
Cut with BalI & BstEII
End fill
Add BglII linkers
5'untranslated
3'untranslated
BglII
SP6
HindIII
dA23dC30
polylinker
P
EcoRI
ori
pSP64T
3280 bp
Amp^r

SECTION 3:2. RESULTS

3:2:1. Tailoring the ricin cDNA clone for expression in heterologous systems.

The expression of ricin cDNA's in heterologous systems has been recognised as being an important contribution to the development of immunotoxins. Such an approach would produce suitably modified toxin molecules which could be characterised in terms of their structure, mechanism of action and physiological effects. (Roberts and Lord 1984).

Earlier manipulations in this laboratory, (carried out by L. Roberts, J. Gould and A. Smith), had generated a number of ricin A chain containing plasmids, some of which were used in the present study.

1) Plasmid pRCL617△4, whose construction is described elsewhere, (O' Hare et al., 1987), in which an Xho I linker immediately precedes the preproricin cDNA sequence commencing at position -74, (Lamb et al., 1985). this construct has an in - frame ATG codon at -72 to -70. Subsequently to this a ricin genomic sequence was published (Halling, et al., 1985). It was evident that the sequence deleted from the cDNA to create pRCL617△4 in fact encoded 10 amino acids forming the N- terminus of the 35 residue signal peptide. In the cDNA, the sequence did not extend far enough to detect the initiating methionine codon.

2) Plasmid M13 mp8 A. The 5' end of the ricin A chain sequence was derived from pRCL617△4 and at the 3' end a stop codon had been introduced by Oligonucleotide- directed mutagenesis immediately

after the A chain coding sequence. This work has been described previously in O' Hare et al., 1987. The ricin A chain cDNA with 3'termination codon in M13mp8 is known as M13mp8A.

3.2.2. Cloning a cDNA sequence encoding ricin A chain with a 3' termination codon in pGEM 1.

To generate a full length ricin A chain cDNA with a 3' termination codon, plasmid pRCL617Δ4 and M13mp8A were cut with restriction enzymes Pst_1 *Cla_1*. The large fragment generated by this digest of pRCL617Δ4 and the small fragment from M13mp8A were gel eluted and ligated to form pUC8RA as in figure 3.2.1.

A Pst_1/Xho_1 fragment from pUC8RA was gel eluted and ligated into pGEM 1 to generate pGEM 1RA. A restiction endonuclease map of pGEM 1 RA is shown in figure 3.2.2. demonstrating insertion of a full length ricin A chain encoding sequence in the correct orientation.

3.2.3. Cloning a cDNA sequence encoding proricin in pGEM 1 and pSP64T.

The transcription / translation vector pSP64T (Krieg and Melton 1984b), (see figure 3.1.3.) , was a gift from Alan Colman , University of Birmingham , U.K. Plasmid pRCL617 4, the source of proricin encoding DNA, was cut with Pst_1 and Xho_1. the ends polished using T4 polymerase (Amersham) in the absence , then the presence of dNTP's (see section 2.5.5.) and the small fragment gel eluted (see figure 3.2.3.). The vector pSP64T was cut with Bgl_II and the ends filled in using T4 polymerase. The eluted proricin encoding fragment

FIGURE 3.2.1. CONSTRUCTION OF RICIN A CHAIN IN pGEM 1

A 1.94kb Pst_1 fragment encoding proricin, (Lamb et al., 1985) was deleted to create pRCL617Δ4, (see text). An 873bp Bam_H1 fragment from pRCL617Δ4 encoding 11 amino acids of the ricin signal sequence, the whole of the ricin A chain and linker region and 5 amino acids of ricin B chain was cloned in M13mp8 to create M13mp8 A. Both pRCL617Δ4 and M13mp8 A were a gift from Dr. L.M. Roberts. A 5' Xho_1 site and 3' termination codon were introduced into the A chain sequence as described in O' Hare et al., (1987).

A ricin A chain encoding fragment was cloned into pUC8 by cutting pRCL617Δ4 with Cla_1 and Pst_1 and eluting the large fragment and cutting M13mp8 A with the same enzymes and eluting the small fragment. The plasmid pUC8 RA was created by ligating the two eluted fragments.

Plasmid pGEM 1 A was created by ligating a gel eluted 873bp Xho 1/Pst_1 A chain encoding fragment from pUC8 RA and ligating this into Sal_1/Pst_1 cut pGEM 1.

□ = coding sequence of ricin A chain cDNA

▨ = coding sequence of ricin B chain cDNA

▧ = linker region

SP6 & T7 = SP6 and T7 RNA polymerase promoters respectively

MCS = multiple cloning sites

Amp^r = Ampicillin resistance gene.

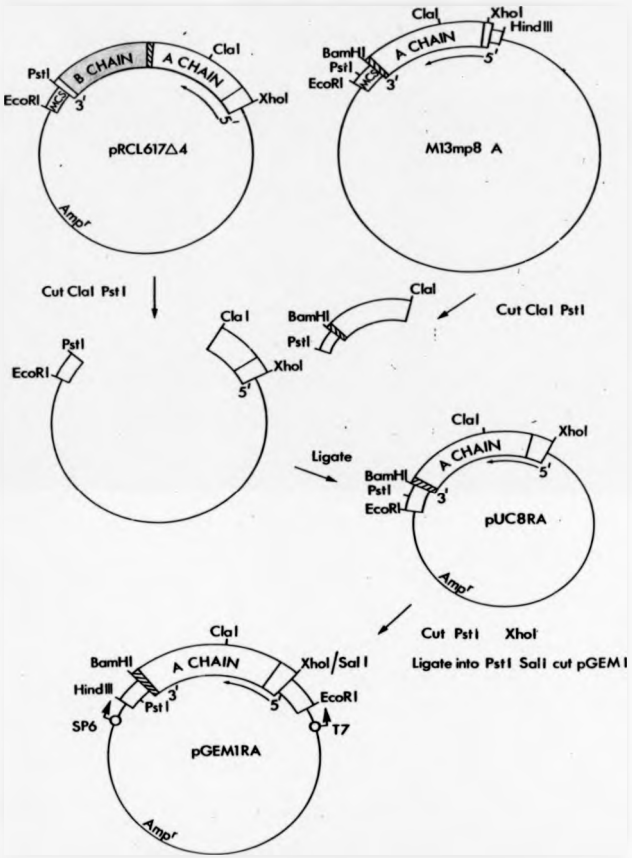
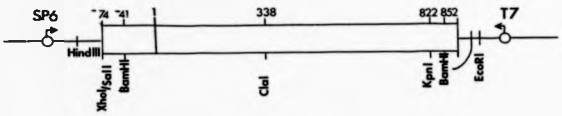
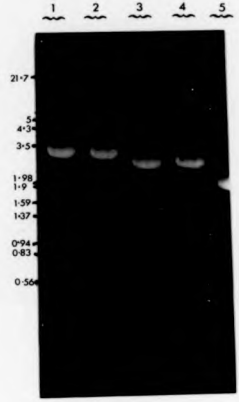

ClaI
XhoI
HindIII
BamHI
PstI
EcoRI
A CHAIN
5'
3'
M13mp8 A
PstI
B CHAIN
A CHAIN
ClaI
EcoRI
XhoI
pRCL617Δ4
Ampr
Cut ClaI PstI
ClaI
BamHI
PstI
Cut ClaI PstI
PstI
EcoRI
ClaI
XhoI
5'
Ligate
ClaI
XhoI
A CHAIN
BamHI
PstI
EcoRI
3'
5'
pUC8RA
Ampr
Cut PstI XhoI
Ligate into PstI SalI cut pGEM1
ClaI
BamHI
A CHAIN
XhoI/SalI
HindIII
3'
PstI
5'
EcoRI
SP6
T7
pGEM1RA
Ampr

FIGURE 3.2.2. RESTRICTION ENDONUCLEASE ANALYSIS OF pGEM1 A.

Plasmid pGEM1 A DNA (1ug), lane 5, was digested with; Kpn 1/Eco RI. lane 4; BamHI. lane 3; Hind III/Cla 1. lane 2; Eco RI/Cla I, lane 1, and the digestion reactions analysed on a 1x TAE agarose gel run at 1.5V / cm for 16 hours. The DNA was electrophoresed on the same gel as shown in figure 3.2.4., the size markers on this gel are Hind III/Eco RI cut lambda DNA and the distance migrated by these fragments is indicated. The gel was stained in 0.1g / ml EtBr for 15min. following electrophoresis. A restriction map of pGEM1 A is shown below for reference. The exact size of the ricin A chain encoding sequence was already known (Lamb, et al., 1985).



pGEM1 = 3kb


1
2
3
4
5
21·7
5
4·3
3·5
1·98
1·9
1·59
1·37
0·94
0·83
0·56

was blunt end ligated into the cut pSP64T, (see figure 3.2.3.) and the orientation of the insert assessed by restriction enzyme digestions as shown in figure 3.2.4.

Proricin encoding cDNA was cloned into pGEM 1 by simply gel eluting the small fragment of an Xho 1 / Pst 1 digest of pRCL617 4 andligating this into Sal 1 Pst 1 cut pGEM 1 to create pGEM1 AB.

3.2.4. Cloning a cDNA sequence encoding ricin B chain with a 5' ricin signal sequence in pGEM BLUE.

This DNA was a gift from P. Richardson and its construction , in vitro expression and analysis are described in Richardson et al., 1988.

3.2.5. Cloning Prepro - alpha factor cDNA in pGEM 2 and Chicken lysozyme cDNA in pSP64.

In using a transcription / translation system it is necessary to have available cDNA's cloned in SP6 / T7 vectors which translate efficiently in a variety of systems. Two clones were obtained for this purpose as positive controls: Chicken Lysozyme cDNA cloned in pSP64 (Melton et al., 1984) was a gift from Alan Colman, whilst prepro - alpha factor from a yeast (Saccharomyces cerevisiae), cDNA library cloned into pGEM 2 was a gift from Dr. J . Rothblatt, EMBL, Heidelberg, FRG. These clones were used throughout the study as positive controls since they are known to translate very efficiently

FIGURE 3.2.3. CONSTRUCTION OF PRORICIN IN pSP64T.

Plasmid pRCL617Δ4 was cut with Pst 1/Xho 1 to release a 1.94kb proricin encoding fragment. The 5' and 3' ends were treated with T4 polymerase, first without, then with, dNTP's, the resultant blunt - ended fragment was ligated into Bgl II cut, end - filled pSP64T to create pSP64T AB.

To create pGEM 1AB, the 1.94kb fragment released from a Pst 1/Xho 1 digest of pRCL617Δ4 was simply gel eluted and ligated into Sal 1/Pst 1 cut pGEM 1, to create pGEM 1AB. (vector not shown).

The orientation of the insert in pSP64T ab was determined as shown in figure 3.2.4.

□ = coding sequence of ricin A chain cDNA

□ = coding sequence of ricin B chain cDNA

□ = signal sequence of ricin A chain

▨ = linker region

MCS = multiple cloning sites

5' and 3' = 5' and 3' untranslated regions of Xenopus β globin cDNA

Amp^r = Ampicillin resistance gene

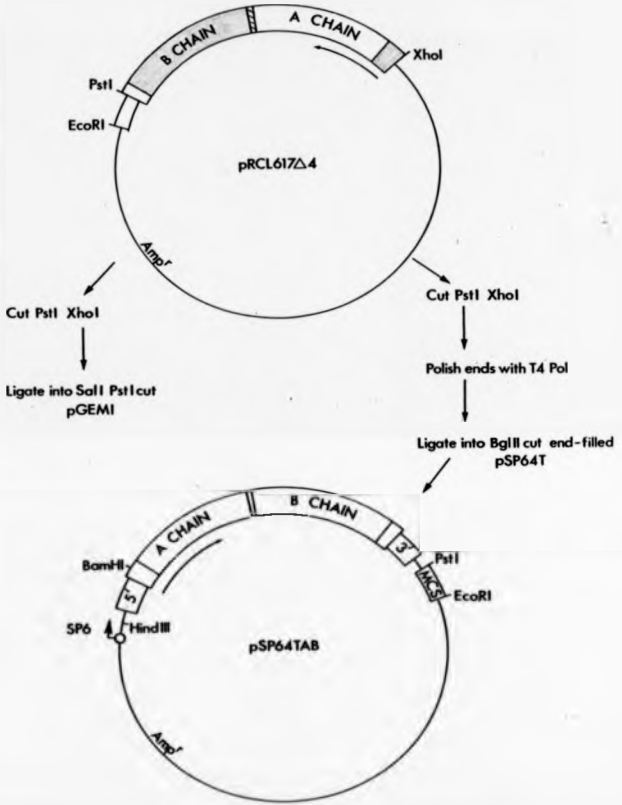
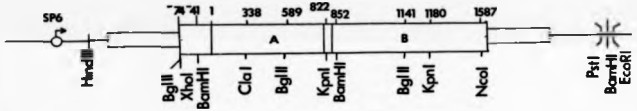
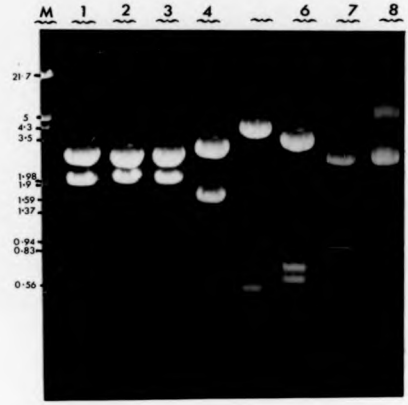

B CHAIN
A CHAIN
XhoI
PstI
EcoRI
pRCL617△4
Amp^r
Cut PstI XhoI
Ligate into SalI PstI cut
pGEM1
Cut PstI XhoI
Polish ends with T4 Pol
Ligate into BglII cut end-filled
pSP64T
B CHAIN
A CHAIN
3'
PstI
MCS
EcoRI
BamHI
5'
SP6
HindIII
pSP64TAB
Amp^r

FIGURE 3.2.4. RESTRICTION ENDONUCLEASE ANALYSIS OF pSP64T AB.

Plasmid pSP64T AB DNA, (1ug), lane 8, was digested with; Bgl II. lane 7; Bam HI. lane 6; Hind III/Cla I. lane 5; Eco RI/Cla I. lane 4; Hind III/Pst I. lane 3; Hind III/Eco RI. lane 2; and Xho I/Eco RI, lane 1. The digestion reactions were analysed on a 1x TAE agarose gel run at 1.5V / cm for 16 hours. The size markers are a Hind III/Eco RI digest of lambda DNA, lane M. The gel was stained in 0.1g / ml EtBr for 15 min. following electrophoresis. A restriction map of pSP64T AB is shown below for reference.



pSP64T = 3kb


M
1
2
3
4
6
7
8
21·7
5
4·3
3·5
1·98
1·9
1·59
1·37
0·94
0·83
0·56

indeed as indicated by the work of Drummond et al., (1985) and Rothblatt et al., (1987), respectively.

Chicken Lysozyme cDNA has been well characterised; Sippel et al.. (1978), Lindenmaier, (1979), Jung et al.,(1980), Land et al.,(1981.,).

The construction of pSP64 Lys+, the lysozyme gene cloned in pSP64 (Melton et al., 1984), is described in Drummond et al., (1985). For transcription, pSP64 Lys+ was linearised using EcoR 1. the resulting 541 base - pair transcripts, when translated should produce a polypeptide of about 14 kDa.

Yeast pre - alpha factor cDNA is a similarly well characterised gene. Rothblatt and co - workers obtained a prepro - alpha factor gene, MF 1, described originally by Kurjan and Herskowitz, (1982). A 602 base - pair HinF 1 - Sal 1 fragment, containing the entire coding region of MF 1 was inserted into the polylinker region of pDS 6. (Stuebber et al., (1984), to produce pDS 6 - 3. (Rothblatt and Meyer , 1986a). The construction of pGEM 2 36. the yeast prepro - alpha factor gene cloned in pGEM 2 is described in Rothblatt et al., (1987).

Plasmid pGEM 2 36, when linearised with Pvu II. and transcribed using SP6 RNA polymerase should produce a 650 base - pair transcript. When translated, an 18.6 kDa polypeptide should be produced.

CHAPTER 4.

In vitro TRANSCRIPTION OF RICIN cDNA SEQUENCES.

SECTION 4:1 INTRODUCTION.

Two methods were tried for the production of microgram quantities of RNA using phage polymerases SP6 and T7, to transcribe cDNA's cloned downstream from the relevant phage promoter. The two methods differed in the way the reactions were set up, the reaction temperature and the processing of the RNA after the reaction was completed. The first method, (method 1), described by Krieg and Melton, (1984), involved the addition, individually, of the separate components of the transcription reaction, namely; DTT, BSA, rATP, rGTP, rCTP, rUTP, Human placental ribonuclease inhibitor, cap analogue, DNA template and the relevant phage polymerase. The reaction mix was incubated at 37°c with 1µl, 1mM GTP added after 30 min. to allow efficient RNA synthesis after initial capping. After the reaction was completed, the RNA synthesised was quantified, phenol extracted and ethanol precipitated in the presence of ammonium acetate, (not sodium acetate) and tRNA as a carrier. RNA was taken up in a suitable volume of sterile distilled water.

The second method, (personal communication from D. Meyer, EMBL, FRG.) was much simpler, as described in section 2:10. A premix containing all the components of the reaction, except for the phage polymerase, cap analogue and ribonuclease inhibitor was made up in bulk and stored at -80°c. Prior to performing the reaction, a master mix was made up containing the premix, plus RNAasein and cap analogue. Equal volumes of this were then added to the linearised template and the reaction started by the addition of the relevant phage polymerase. The reaction temperature was 40°c, even though Krieg and Melton point out the likelihood of spurious transcription products at this temperature, (Krieg and Melton, 1986). As in the former method, 1µl

of 8mM GTP was added after 30 min. to ensure efficient RNA synthesis. At the end of the reaction, there was no phenol extraction step, reactions were simply quick frozen in liquid nitrogen and stored, ready for use at -80°c.

This chapter compares the efficiency of these two methods for RNA synthesis and describes the sizing, assessment of integrity and quantification of transcripts synthesised.

SECTION 4:2 RESULTS.

Figure 3.1.1. shows a schematic representation of a typical transcription reaction, (for method see section 2:10). Both methods 1 and 2, introduced above, were tested to compare and contrast their ability to efficiently transcribe a variety of ricin cDNA's cloned in SP6 / T7 vectors.

No difference was noted in the translational ability of phenol extracted and non - extracted RNA template, indeed, the non - extracted RNA translated more efficiently in a wheatgerm lysate.

After 1 hours incubation, 1µl of the in vitro transcription reaction was denatured in denaturing buffer at 65°c for 10 minutes. Samples were loaded immediately on a 1.5% agarose formaldehyde gel (see section 2:4:3.) in 1x MOPS buffer. The gel was run at 40mA for 2 hours, vacuum dried at 60°c for 1 hour and exposed to Fuji RX X - Ray film for 30 minutes at -80°c. Figure 4.2.1. is an autoradiograph showing the typical exposure pattern observed for ricin cDNA transcription products. Lane M is a series of molecular weight markers transcribed from cDNA's kindly donated by L. Tabe; Veg 1 (2kb), Histone 2B (1.85kb), Histone 1 (1.6kb), and Lys' (0.5kb). Lane 1

FIGURE 4.2.1. TRANSCRIPTION OF PRORICIN, RICIN A CHAIN AND RICIN B CHAIN

Lanes 1, 2 and 3 represent transcripts prepared by the " premix " method described in section 2:10. Transcripts run in lane 4 and the marker transcripts, (M), were prepared by adding components of the transcription reaction individually as described by Krieg and Melton (1984).

Lane 1 pSP64T AB template.

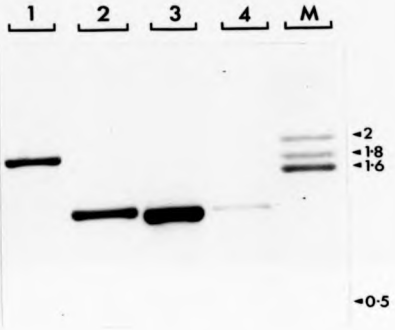
Lane 2 pGEM BLUE preB template

Lane 3 pGEM1 A template

Lane 4 pGEM1 A template

M Marker transcripts prepared individually of templates encoding chicken lysozyme, (0.5kb), Histone 1, (1.6kb), Histone 2B, (1.8kb) and Vag 1, (2kb), prepared from DNA provided by Prof. A Colman, University of Birmingham.

In each case, 0.5µl of 44TBq / mmol [^{32}P] UTP was added to the transcription reaction. At the end of the reaction, 5µl of the reaction mix was denatured, loaded on a 1.5% agarose formaldehyde gel, run for 3 hours at 40mA, dried down and exposed to Fuji RX X - Ray film at -80°c for 30 minutes as described in section 2:4:3.


1
2
3
4
M
2
1·8
1·6
0·5

is 1ul of proricin RNA transcribed from the SP6 promoter of pSP64T. Lane 2 is 1ul of pre B chain RNA (Richardson et al., 1988), transcribed from the SP6 promoter of pGEM BLUE. Lanes 3 and 4 are ricin A chain RNA transcribed from the T7 promoter of pGEM 1 using method 2 (lane 3), and method 1 (lane 4).

Several features are immediately obvious;

1) Significant quantities of RNA were synthesised from all cDNA's whether transcribed using SP6 or T7 polymerases. In other words, both polymerases function efficiently in the same buffer.

2) Transcription of ricin A chain cDNA was more efficient using method 2 than method 1. Thus not only was this method easier to perform, it was noticeably more efficient. This effect was repeatedly observed and hence, after these preliminary experiments, all transcriptions were performed using method 2.

3) RNA synthesised by both methods was undegraded.

4) A premix need only be made once. When aliquoted into 50ul volumes, one does not have problems induced by repeated freeze thawing of unstable solutions. A premix made was stored for over two years and was still active

The sizes of transcripts was estimated from the autoradiograph as being comparable with that predicted from the sizes of the cDNA's this gave sufficient reassurance that transcription had started and terminated at the correct places. The exact size of the cDNA was known in each case (Lamb et al., 1985), and so an accurate evaluation of transcript size was not considered essential. After this preliminary study, assessment of the integrity of transcripts was by translation in a wheatgerm lysate followed by polyacrylamide gel electrophoresis. By this means, analysis of cDNA's was greatly accelerated since SDS - Polyacrylamide gel electrophoresis of wheatgerm translation products

this means, analysis of cDNA's was greatly accelerated since SDS - Polyacrylamide gel electrophoresis of wheatgerm translation products was an integral part of the analysis of mutant cDNA's (see chapters 7 to 9).

Where direct comparison of different cDNA's transcribed in vitro was necessary (chapters 7 - 9), it was essential to quantify the reaction so that an equal amount of each RNA species could be added to the translation system for assay (chapter 6). This was normally performed using ^{3}H - UTP. Quantification of transcription reactions was as follows:

2µl of the transcription reaction was spotted onto DE81 paper and was washed twice in 10% TCA with 20mM $Na_4P_2O_7$, for 5 min. each, on ice, twice in absolute ethanol briefly and once briefly in ether. Discs were then air dried and counted in 4ml of Beckman EP scintillant on an LKB 1212 scintillation counter. A typical reaction normally gave 1.5 - 2 x 10^{6} cpm.

Calculation of the amount of RNA synthesised was as follows;

1. The concentration of cold UTP in the reaction was 500uM (500nmoles ml^{-1})

Thus in a 20µl reaction there are

$$\frac{500 \times 20}{1000} = 10\text{nmoles cold UTP}$$

2. There are also 10µCi ^{3}H - labelled UTP containing 1.32 x 10^{7} cpm

Thus the amount of UTP incorporated into TCA precipitable RNA is equal to

$$\frac{\text{TCA precipitable counts}}{\text{N°. counts in 10µCi } ^{3}H \text{ UTP}} \times \text{amount cold UTP}$$

$$= \frac{1.5 \times 10^{6}}{1.32 \times 10^{7} \times 10} \text{ to } \frac{2 \times 10^{6}}{1.32 \times 10^{7} \times 10}$$

= 1.1 nmoles to 1.5 nmoles UTP incorporated into TCA precipitable RNA

The amount of RNA synthesised can be calculated from this by multiplying this figure by the average molecular weight of a ribonucleotide, (340), and the number of bases, (4);

$$1.1 \times 340 \times 4 = 1496 \text{ ng} = 1.5\mu g$$

$$\text{to } 1.5 \times 340 \times 4 = 2040 \text{ ng} = 2.0\mu g$$

When 1µl of a 20µl reaction mix was added to a cell free translation subsequently, effectively 75 - 100ng of RNA was being added.

In this calculation, the amount of UTP contributed by the labelled ribonucleotide was not considered since it can be assumed to be negligible compared to the cold UTP added. Tritiated UTP has a specific activity of 52Ci / mmole (52µCi nmole^{-1}). A total of 10µCi was added per reaction; equivalent to 0.19 nmoles UTP, being 1/50th the amount of cold UTP and can be regarded as negligible

SECTION 4:3 SUMMARY

Microgram quantities of undegraded RNA of the predicted size was synthesised, repeatedly in vitro from a number of ricin encoding cDNA's cloned in transcription vectors.

Two methods were tried, one was found to be significantly more efficient than the other and was therefore adopted for the purposes of

this study since it introduced fewer variables and offered several major advantages over the former;

1. The most significant advantage from the point of view of this study, in which cDNA's encoding mutant and wild type genes were to be compared, is one of uniformity. Equal amounts of similarly purified DNA template with mutant or wild type genes cloned downstream from the same phage polymerase promoter transcribed in identical volumes of the same master mix should, in theory, be transcribed with comparable efficiency. This is of crucial importance to an experiment where the activity of polypeptides translated from different messages are to be compared as in this study. (see chapters 7 - 9).

2. The second advantage is one of speed. The reactions are easily handled without the need for time consuming phenol extractions during which template is inevitably lost. This is of importance where a number of reactions are to be performed simultaneously since they can be started and stopped within seconds of each other.

3. In the present study, more template was produced by method 2.

CHAPTER 5.

In vitro TRANSLATION OF RICIN cDNA CONSTRUCTS.

SECTION 5:1. INTRODUCTION

5:1:1. in vitro TRANSLATION SYSTEMS.

The use of cell - free protein synthesising systems has been a routine technique in molecular biology labs for many years . Several such systems exist; of these, the rabbit reticulocyte and wheatgerm lysates have been the most widely used since they are easy to prepare, are readily available in large quantites at low cost and show very high translational efficiency. The preparation and optimisation of the rabbit reticulocyte lysate is covered in detail by Clemens, (1984), whilst the wheatgerm lysate, although originally described by Roberts and Paterson, (1973), was improved by Anderson et al., (1983). It is the latter method which has been adopted in this work.

In addition to the above systems, extracts from a variety of eukaryotic cell types have been prepared for the translation of exogenous mRNA's, or in order to study aspects of the regulation of protein synthesis. For example, Krebs II mouse ascites cells have been widely used as a source of a cell - free system which will translate added cellular and viral mRNA's, (Mathews, 1972). Extracts from mouse L - cells, Ehrlich ascites tumour cells, HeLa cells and Chinese Hamster Ovary (CHO) cells exhibit similar properties (Mathews and Korner, 1970, Crystal et al., 1974, Haywood and Rourke, 1974, Marcus et al., 1974, Schimke et al., 1974, Schutz et al., 1974, Villa - Komaroff et al., 1974). Recently, yeast has emerged as an organism with great potential for the elucidation of cell biological

phenomena. Yeast lysates have been successfully used to translate efficiently and faithfully, a variety of exogenous mRNA's, (Gamier et al., 1979, Tuite et al., 1980, Chanda and Kung, 1983) and are reviewed by Tuite and Plesset, (1986). The lysate used in this work was a gift from D . Meyer, EMBL, FRG, and was prepared according to the method of Rothblatt and Meyer, (1986a and 1986b).

Cell - free translation systems have held an intimate association with attempts to elucidate the mechanism of action of ricin and related ribosome inactivating proteins over the last 15 years. The present work represents a unique exploitation of this relationship.

With the advent of efficient and convenient in vitro techniques for producing microgram quantities of mRNA described in section 3.1., it has been postulated that cell - free systems may be developed for small - scale synthesis of important polypeptides, (Riordan, 1987). Here, I have attempted to do this in the case of ricin and to further establish that any ricin A chain produced has full biological activity.

SECTION 5:2. RESULTS

5:2:1. Translation in wheatgerm lysate.

Transcripts were made from cDNA clones encoding ricin A chain, ricin B chain, proricin, chicken lysozyme and yeast prepro - alpha factor using the SP6 / T7 in vitro transcription system described in section 4.2. 1µl of each transcription reaction was incubated in the wheatgerm system as described in section 2:10:1 PAGE

and fluorography of 5ul samples was carried out exactly as in section 2:12:1. Immunoprecipitation of translation products was carried out exactly as in section 2:12:2, results are shown in figure 5.2.2.

Figure 5.2.1. shows an autoradiograph indicating the relative sizes of ^{35}S - labelled products in wheatgerm alongside a set of standard ^{14}C - labelled molecular weight marker proteins (Amersham). The molecular weights of the ricin cDNA translation products was estimated to be the same as that previously observed and published.

The size of ricin A chain (31 kDa), correlated well with the size predicted from non - glycosylated recombinant A chain, (O' Hare et al., 1987). The size of ricin B chain correlated exactly with the size predicted from non - glycosylated B chain, (Richardson et al., 1988). For proricin there are no examples to date , of recombinant protein for comparison . The estimated size of deglycosylated , non - reduced whole preproricin from the cDNA (Lamb et al.,1985) was roughly 65 - 67 kDa, the same as that produced in the wheatgerm system. Thus ricin cDNA constructs transcribed and translated in vitro produce polypeptides of the predicted sizes .

Chicken lysozyme appeared as a protein of 16 kDa , similar to the size observed by Krieg et al., (1984) . Prepro - alpha factor appeared as a protein of 20 - 25 kDa , similar to the size observed by Rothblatt et al., (1987).

5.2.2. Translation in rabbit reticulocyte lysate.

The same batch of transcripts were translated in laboratory prepared rabbit reticulocyte lysate . (See section 2:10:2.).

FIGURE 5.2.1. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS OF WHEATGERM TRANSLATION PRODUCTS

1µl of each transcription reaction was added to a wheatgerm lysate and the reaction incubated at 28°c for 1 hour as described in section 2:10:1. SDS - PAGE, fluorography and autoradiography of 5µl samples of each reaction run on a 10% SDS - Polyacrylamide gel was carried out exactly as described in section 2:12. Products of translation were as follows:

Lane 1 Proricin translated from pSP64T AB RNA

Lane 2 Ricin A chain translated from pGEM 1 A RNA

Lane 3 Ricin B chain translated from pGEM BLUE preB RNA

Lane 4 Yeast prepro alpha factor translated from pGEM2 36 RNA

Lane 5 Chicken lysozyme translated from pSP64 Lys RNA

M ^{14}C - labelled molecular weight markers, (Amersham).

Autoradiography was for 3 hours at room temperature, the marker lane was exposed for 12 hours.

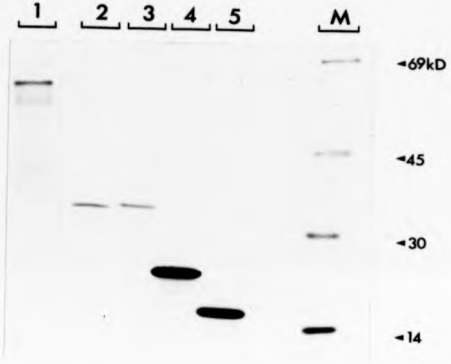

1
2
3
4
5
M
69kD
45
30
14

FIGURE 5.2.2. IMMUNOPRECIPITATION OF PRORICIN, RICIN A CHAIN AND RICIN B CHAIN TRANSLATED IN A WHEATGERM LYSATE.

5µl of the reactions shown in figure 5.2.1. were immunoprecipitated as described in section 2:11:2. The immunoprecipitated translation products and the remaining 2.5µl of the reaction mix were run on a 10% SDS - Polyacrylamide gel for 12 hours, fluorographed and autoradiographed as described in section 2:12.

Lane 1a Immunoprecipitated proricin

Lane 1b proricin

Lane 2a Immunoprecipitated pre B chain

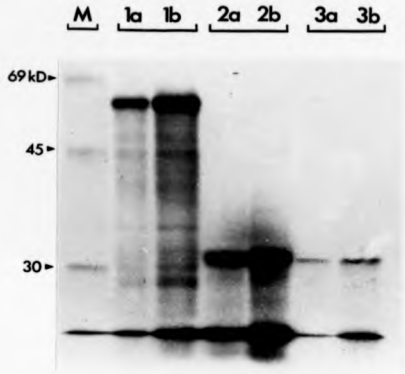
Lane 2b pre B chain

Lane 3a Immunoprecipitated ricin A chain

Lane 3b ricin A chain

M ^{14}C labelled molecular weight markers (Amersham).

Autoradiography was for 12 hours at room temperature.


M
1a
1b
2a
2b
3a
3b
69kD
45
30

The electrophoretic mobility of translation products was assessed by PAGE followed by fluorography and autoradiography. (See section 2:12:1.) . The size of polypeptides translated was not found to differ from that observed in the wheatgerm system, (data not shown) . For a typical reticulocyte lysate translation profile of ricin A chain and ricin B chain see figure 6.2.2., lanes 4 and 2 respectively, and figure 7.2.2., lanes 5 and 3 respectively, whilst for that of proricin see figure 6.2.2., lane 3.

The most striking feature however, of the reticulocyte lysate reactions is the greatly reduced synthesis of ricin A chain compared to that in the wheatgerm system. Figure 5.2.3. demonstrates this by a comparison of wheatgerm and reticulocyte lysate translations of ricin A chain, ricin B chain and proricin. The efficiency of translation of each polypeptide is indicated by the amount of labelled methionine incorporated. It is seen that whilst ricin B chain and proricin accumulate to a significant level in both systems, the amount of ricin A chain which accumulates in the reticulocyte lysate is negligible compared to that in the wheatgerm system. It should be noted, also, that translation in both systems used the same batches of mRNA, incubated for exactly the same length of time. Possible reasons for this discrepancy are discussed later in this chapter, and the ideas developed from this discussion are pursued in detail in later chapters.

5:2:3. Translation in yeast lysate.

Translations of ricin A chain, B chain and proricin were carried out as in section 2:10:3. SDS - PAGE, fluorography and autoradiography were performed as above. It was found to be

FIGURE 5.2.3. THE INCORPORATION OF LABELLED METHIONINE INTO RICIN A CHAIN, RICIN B CHAIN AND PRORICIN DURING TRANSLATION IN THREE *In vitro* SYSTEMS

Ricin A chain, ricin B chain and proricin were translated in a wheatgerm, rabbit reticulocyte and yeast lysate.

5ul of each translation was run on a 10% SDS Polyacrylamide gel in which the Bis - Acrylamide had been replaced by DATD (BIORAD). Labelled bands were detected by autoradiography then cut out and dissolved in 2% Periodic acid. Beckman EP scintillant was added and incorporation of labelled methionine into each polypeptide measured as counts per minute on an LKB scintillation counter.

Ricin transcript	Wheatgerm cpm	Retic.Lys. cpm	Yeast Lys. cpm
A chain	375,428	14,137	164
B chain	436,719	72,608	278
Proricin	725,905	81,595	125

impossible to detect the accumulation of any protein at all by autoradiography or by assessing the level of incorporation of labelled methionine into TCA precipitable protein (see figure 5.2.3.). Reasons for this are explored in detail in the remainder of this chapter.

Ricin A chain, ricin B chain and proricin encoding RNA translated efficiently in wheatgerm lysate, less so in a rabbit reticulocyte lysate and were not detected by SDS - PAGE, fluorography and autoradiography in a yeast lysate. Significant differences were noticed in the efficiency of translation of ricin A chain in the wheatgerm and rabbit reticulocyte lysates and this prompted a detailed investigation of possible causes.

Data presented in sections 5:2:1 and 5:2:2 revealed a marked difference in the efficiency of translation of ricin A chain between that in the rabbit reticulocyte lysate and in a wheatgerm lysate. It was impossible to detect stimulation of methionine incorporation above background by added ricin encoding transcripts in the yeast lysate. Clearly marked differences exist between the three systems, either in their ability to support translation or in their response to added ricin A chain template and translation from this. Differences in the translational efficiency between the added transcripts must also exist There are many possible reasons why translation of one mRNA should be less efficient than another, or why the products of one mRNA species should accumulate to different extents in different systems. Protein synthesis is, after all, an extremely complex system with many contributory components and requirements .

Reasons for this difference, however, fall into two main categories .

1. Properties of the lysate :

Lack of ricin A chain translation in the yeast lysate and poor translation in the rabbit reticulocyte lysate could be due simply to deficiencies in components in the lysate necessary to support efficient translation . Such a problem is widely encountered and is simply corrected by adjusting the levels of components in the lysate until the system is optimized for translation of a particular mRNA. The analysis of possible deficiencies in components is approached experimentally below.

2. Properties of the mRNA's or the polypeptides they encode.

5:2:4. Analysis of possible deficiencies of components in the lysate.

The composition of the reaction mixtures for the three cell - free systems varies. Table 5.2.1. shows a comparison of the concentration of components in each lysate system.

If differences are observed in the efficiency of translation of a particular message from one lysate to another it may be a simple matter of adjusting conditions for translation of that particular message to enhance the efficiency of its translation. By this means, the relative contributions of each of the components can be determined and the benefit to be gained by alteration to these recommended levels assessed. Although the concentrations of components are seen to vary, on the whole, there are no significant differences between the three systems. However, it is known that in cell - free translation systems efficient translation of exogenous mRNA is highly dependent on an optimal potassium and magnesium ion concentration. Dr.David

Lightfoot, (University of Warwick) analysed in some detail, the effect of Magnesium ion concentration on translation of glutamine synthetase (GS), encoding mRNA produced by SP6 transcription in a wheatgerm lysate system. (Lightfoot, 1988). A very sharp magnesium optimum peak at 1.5mM was noted. Higher concentrations (up to 3.0mM), reportedly affected peptide chain elongation and termination. Purified RNA's to GS subunits γ, δ, and α had the same optimum whilst purified pre plastocyanin RNA (Smeekens, 1986) had an optimum at 3.5mM Magnesium. Clearly in a system where ricin A chain is to be translated efficiently optimisation is essential and may be the cause of poor translations in a non - optimised yeast lysate. It was considered that adjustment of levels of these salts would lead to more efficient translation of ricin A chain in yeast lysates in particular. In the following subsection I have presented measures taken to do so.

5:2:4:1. Optimisation of potassium and magnesium ion concentrations in the yeast lysate.

In setting up the translation systems it was found that the concentrations of magnesium and potassium ions indicated in table 5.2.1. were optimal for all messages translated in the wheatgerm and reticulocyte lysate systems. In the yeast lysate system, translation of ricin A chain mRNA was so conspicuously poor that it was considered necessary to investigate salt concentration in more detail.

Optimisation was carried out simply by varying one salt concentration, whilst keeping the other at the recommended level. Two messages were translated. Yeast prepro - alpha factor mRNA (see section 3.3.5.) was used as a control since it is known to translate efficiently in a yeast lysate made as in section 2:10:2., as described

by Rothblatt and Meyer, (1986). Ricin A chain message was also translated under the same conditions. Table 5.2.2. shows the range of potassium and magnesium ion concentrations used in this experiment .

TABLE 5.2.1. Concentrations of components in the three lysate systems

COMPONENT	WHEATGERM	RABBIT RETICULOCYTE	YEAST
HEPES (KOH)	20mM pH 7.6	-	25 mM pH 7.4
ATP	1mM	1mM	0.5mM
Creatine Phosphate	8mM	7mM	25mM
Creatine Phosphokinase	40ug / ml	100ug / ml	80ug / ml
Spermine	30ug / ml	-	-
DTT	2mM	-	2mM
GTP	20uM	200uM	100uM
19 amino acids (each)	25uM	50 - 200uM	25uM
Magnesium acetate	20uM	2mM	3mM
Potassium acetate	120mM	75mM	300mM
Calcium chloride	-	2mM	0.1mM
Haemin	-	10 - 200uM	-
TRIS -HCL pH 7.6	-	10mM	-
Glucose	-	3mM	-

TABLE 5.2.2. Potassium and magnesium ion concentrations used in optimising the yeast lysate system for translation of ricin A chain encoding RNA

Reaction No.	[Mg++]	[K+]
1	3mM	100mM
2	3mM	150mM
3	3mM	200mM
4	3mM	250mM
5	3mM	300mM
6	3mM	350mM
7	3mM	400mM
8	0mM	300mM
9	1mM	300mM
10	2mM	300mM
11	3mM *	300mM *
12	4mM	300mM
13	5mM	300mM
14	6mM	300mM

* 3mM Mg++ and 300mM K+ are the recommended optional levels of both ions (Rothblatt and Meyer, 1986) .

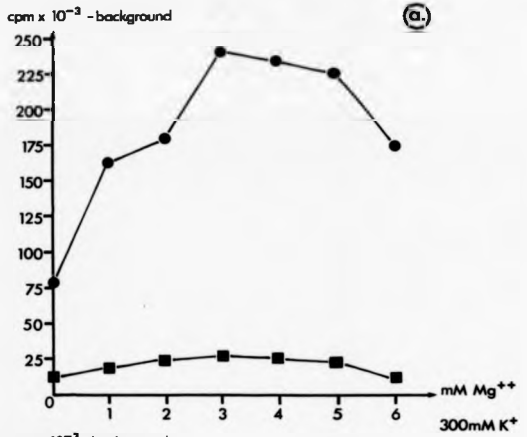
A separate 25ul reaction was set up for reactions 1 - 14 , above , at 25° C as described in section 2:10:2. for 1 hour. At the end of this time 2ul aliquots of the reaction were spotted on Whatman No. 1 1cm square discs and TCA precipitable counts measured in 4ml Beckman EP scintillation fluid on a LKB Minibeta 1212 scintillation counter. Data are presented graphically in figures 5.2.4.a. and 5.2.4.b.

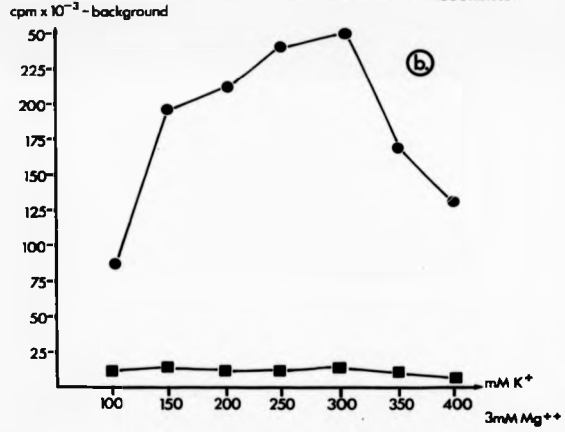
FIGURE 5.2.4. OPTIMISATION OF [K+] AND [Mg+] IN A YEAST LYSATE FOR THE TRANSLATION OF PREPRO ALPHA FACTOR AND RICIN A CHAIN MESSAGES : GRAPHICAL REPRESENTATION.

Fourteen 25µl reactions were set up as described in table 6.2 and the reactions started by the addition of 1µl of ricin A chain message. A separate 14, 25µl reaction mixtures were set up identically apart from the addition of 1µl of prepro alpha factor RNA. Reactions were incubated at 25°c for 1 hour at the end of which, 2µl aliquots were spotted onto 1cm Whatman N°.1 discs and label incorporated into protein TCA precipitated and counted on an LKB 1212 minibeta scintillation counter as described in section 2:11:1. Figure 5.2.4a represents the variation of [Mg^{++}] whilst keeping [K^{+}] constant at 300mM. Figure 5.2.4b represents the variation of [K^{+}], whilst keeping [Mg^{++}] constant at 3mM.

■ = ricin A chain message.

● = prepro alpha factor message in both 5.2.4a and b.


cpm x 10^{-3} - background
(a.)
250
225
200
175
150
125
100
75
50
25
0
1
2
3
4
5
6
mM Mg^{++}
300mM K^+


cpm x 10^{-3} - background
(b.)
50
225
200
175
150
125
100
75
50
25
100
150
200
250
300
350
400
mM K^+
3mM Mg^{++}

It is evident that the recommended concentrations (3mM Mg++ and 300mM K+) were optional for translation of prepro - alpha factor mRNA. In contrast, added ricin A chain transcripts did not significantly stimulate translation above background levels in the yeast lysate whatever the concentration of magnesium or potassium used. To check whether or not the unusually low counts obtained for ricin A chain translation were due to an error during the TCA precipitation and scintillation counting procedure, 5ul samples of each of reactions 8 - 14 were run on a 10% polyacrylamide gel, fluorographed and autoradiographed as in section 2:12. Figures 5.2.5.a. and 5.2.5.b., lanes 1 - 7 represent reactions 8 - 14 (See Table 5.2.1.), for prepro - alpha factor mRNA and ricin A chain mRNA in yeast lysate, respectively. Figure 5.2.5.a. lanes 1 - 7 shows that prepro - alpha factor RNA translates in a yeast lysate system even when Mg++ concentrations are non - optimal. Similarly, as in the graphical representation of data in figures 5.2.4.a. and b., there was no visible translation of ricin A chain RNA, a band of 31kDa being absent from lanes 1 - 7 in figure 5.2.5.b. The possibilities existed that the SP6 transcript encoding ricin A chain was degraded or that it had been transcribed in such a way as to render it incapable of translation. These were simply tested as follows:

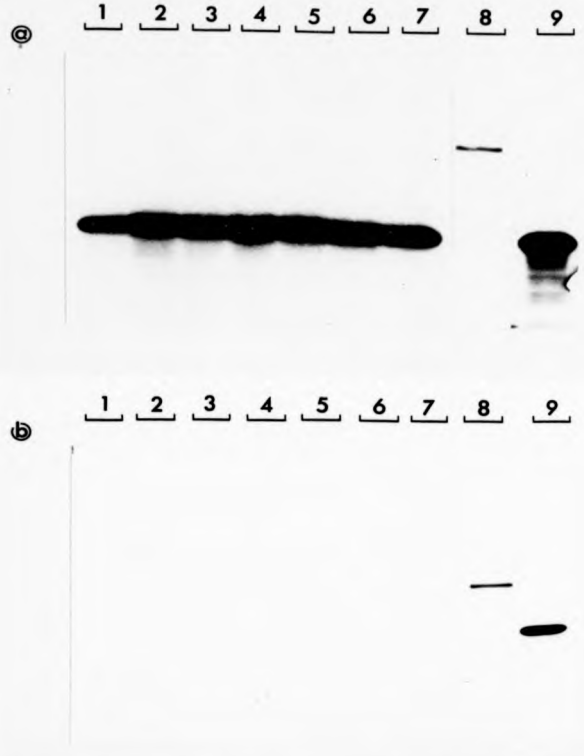
1. Both the batches of transcripts were labelled with ^{32}P - labelled UTP, these were detected in the lysate by denaturing 10ul of the translation reaction and electrophoresis on a 1.5 % agarose / formaldehyde gel. The integrity of the RNA was analysed by autoradiography of the dried gel. (Figures 5.2.5.a. and b., lanes 8.). It is evident that after one hour of translation in a yeast lysate the transcripts encoding both prepro - alpha factor and ricin A chain are intact.

FIGURE 5.2.5. OPTIMISATION OF [Mg^{++}] IN A YEAST LYSATE FOR THE TRANSLATION OF RICIN A CHAIN AND PREPRO ALPHA FACTOR MESSAGES: SDS - PAGE ANALYSIS. ANALYSIS OF RNAase ACTIVITY AND THE TRANSLATION OF THE SAME MESSAGES IN A WHEATGERM LYSATE.

From the remainder of reactions 8 to 14 (table 5.2), of ricin A chain and prepro alpha factor translations, 5ul was run on a 10% SDS - Polyacrylamide gel, fluorographed and autoradiographed as described in section 2:12. Lanes 1 to 7 of figure 5.2.5a represent reactions 8 to 14 showing the response of prepro alpha factor translation to a variation in [Mg^{++}] from 0 to 6mM. Lanes 1 to 7 of figure 5.2.5b represents the same response of ricin A chain message.

To test whether or not the lack of ricin A chain translation, (lanes 1 - 7 fig. 5.2.5b), was a result of RNA degradation, 5ul samples were taken from one of each of the two sets of reactions, chosen at random and run on a 1.5% agarose formaldehyde gel as described in section 2:4:3 as shown in lanes 8a and b.

To check whether the same batch of transcripts could translate in a wheatgerm, 1ul of each species of RNA was translated, electrophoresed and processed as described in section 2:11:1 as shown in lanes 9a and b.


@
1 2 3 4 5 6 7 8 9
b
1 2 3 4 5 6 7 8 9

2. The ability of the transcripts to direct translation was demonstrated by incubating samples of the same batch of transcripts in a wheatgerm translation system and analysing the products by SDS - PAGE and autoradiography as shown in figures 5.2.5.a and b. lanes 9. It was evident that these RNA's could stimulate efficient translation, albeit, in a different system.

Thus, even though the RNA was capable of directing translation, and, after one hours incubation in a yeast lysate at 25°c it was not degraded, it is still not translated efficiently. Adjustment of Mg++ and K+ concentrations had no effect either, as indicated by the fact that prepro - alpha factor RNA, which shows the same salt requirements as ricin A chain in a wheatgerm lysate, translated efficiently under the recommended conditions.

Many other factors govern the synthesis of a protein in an in vitro system; some are more easy to analyse than others.

One possibility is that codon usage differs between the wheatgerm and yeast lysates. At the time of this work there were no examples of plant mRNA's being translated in a yeast lysate. (M. Tuite , personal communication). It is possible that the correct aminoacyl tRNA's for translation of a plant message in a yeast lysate are not present. Two measures were taken to reject this argument :

1. The yeast lysate system was supplemented with tRNA extracted from wheatgerm (Sigma) in addition to that already in the system. Even with plant tRNA available translation of ricin A chain was not detected (data not shown).

2. A number of plant mRNA's were prepared from cDNA's coding for preplastocyanin and ferredoxin (gifts from C . Robinson). These

were translated efficiently in a yeast lysate which was not supplemented with plant tRNA (See section 5:2:5:3. and 5:2:5:6.).

Thus it would appear that problems associated with codon usage are not the cause of failure of ricin A chain to translate in yeast lysates since other plant messages can do so efficiently and plant tRNA supplementation has no effect.

These data imply that in each lysate, all the necessary components for translation are present; differences noted in the efficiency of translation of various messages are therefore not due to any deficiency in the lysate itself. This must mean, therefore , that such differences are a direct result of properties of the mRNA's themselves or properties of the proteins they encode.

5:2:5. Analysis of the factors affecting efficiency of translation of mRNA's.

Section 5:2:4. provided evidence to suggest that differences between wheatgerm, reticulocyte and yeast lysate translation of ricin A chain encoding RNA are due to properties of the RNA or the polypeptide expressed from it. A number of factors are known to regulate translation of exogenous RNA in vitro. These include:

1. Presence or absence of a 5' cap structure,
2. Presence or absence of a 3' polyadenylated tail,
3. Degradation of the mRNA,
4. Poor initiation of translation,
5. Incomplete peptide chain elongation,
6. Protein degradation,

7. Toxicity of the protein synthesised.

Each of these factors was investigated experimentally, where possible, and the implications of their involvement in the inhibition of ricin A chain translation assessed.

5.2.5.1. 5' Capping.

The normal product of a transcription reaction contains a triphosphate group at the 5' end of the molecule. For some purposes, however, it is necessary to cap the 5' end of the synthetic transcript. For example, a 5' cap is required for efficient splicing of pre - mRNA's when using cellular extracts in vitro. (Konarska et al., 1984, Krainer et al., 1984,). A 5' cap is essential for the stability of mRNA in injected oocytes. (Green et al., 1983, Krieg and Melton, 1984.b., Drummond et al., 1985,). Furthermore, the presence of a 5' cap increases the translational activity of synthetic mRNA in cell - free translation systems, (Paterson and Rosenberg, 1979, Krieg and Melton, 1984.b., Mead et al., 1985.).

However, in all the translations performed, the mRNA produced by SP6 / T7 transcription had a 5' cap structure. This was added during the transcription reaction, the cap analogue, m$_7$G(5')ppp(5')G, being added in a ten - fold excess over the GTP concentration. Making the assumption that different transcripts are capped to the same extent, this was not considered a likely cause of poor translation.

5.2.5.2. Polyadenylation of RNA.

It is known that polyadenylation of synthetic RNA's enhances their long term stability when injected into Xenopus oocytes and monkey CV1 cells, (Drummond et al., 1985) or HeLa cells (Huez et

al., 1981). However, in both reticulocyte and wheatgerm lysates, the polyadenylation in vitro of four different mRNA's caused a 70 % decrease in their translation (Drummond et al., 1985). It is speculated that the observed inhibition is due to absorption of crucial translational components by the poly (A) tracts present. Ricin A chain cDNA was the only clone used which did not posess a poly (A) tail. If the presence of a poly (A) tail is inhibitory to in vitro translation of that mRNA as reported by Drummond and colleagues, then this cannot be seen as a possible reason for ricin A chain not to be translate, quite the opposite to the observed should be expected in theory.

5:2:5:3. Degradation of RNA.

Destruction of the synthetic RNA in the cell free systems is an obvious and easily tested cause of lack of translation. Data presented in figures 5:2.5.a. and b., lanes 8, indicated that after 1 hours translation in a yeast lysate 32P labelled transcripts encoding prepro - alpha factor and ricin A chain respectively were not degraded. A more thorough investigation into the stability of transcripts in yeast and wheatgerm lysates was proposed.

It is known that, particularly in yeast lysates, specific nucleases are present which affect the cell - free translation of RNA, (Herrera et al., 1979). The activity of these may be limited, however, by incubating the translation below 20° c, in which case, the nucleases are inhibited. However, no difference was observed when prepro - alpha factor RNA was translated below, (18°c), at, (20°c), or above, (25°c), this temperature. indeed all yeast lysate translations were carried out as recommended by D. Meyer at 25°c.

A similar range of incubation temperatures was tried for ricin A chain translations. No detectable stimulation above background incorporation of labelled methionine into TCA precipitable counts was observed at the three temperatures, (See table 5.2.3.).

TABLE 5.2.3. The effect of temperature on the translation of ricin A chain and prepro - alpha factor RNA in yeast lysate.

	TCA precipitable counts for each RNA species		
Temperature	- factor	A chain	No RNA
18°c	153409	19000	15135
20°c	144011	18421	17929
25°c	180257	18816	14490

1µl of a transcription mix was added to a yeast lysate set up as in section 2.10.2., and incubated for 1 hour. 2µl aliquots of each reaction were spotted onto 1cm square Whatman No.1 discs, TCA washed and counted in 4ml Beckman EP scintillation fluid in an LKB 1212 scintillation counter. TCA precipitable counts are expressed as counts per minute.

RNA degradation due to temperature induced stimulation of ribonucleases in the yeast lysate is not, therefore, suspected.

RNA degradation may be due to RNA species specificnucleases, which may recognise a particular feature of individual RNA species. For instance, nucleases may be present in a yeast lysate which recognise particular tertiary structural features, such as hairpin loops or particular sequences on the template, rendering individual templates open to their attack. If such nucleases exist one could predict,

therefore, that different RNA templates would have characteristic susceptibilities to such attack. The result being differential expression of different templates. If ricin A chain encoding RNA were particularly sensitive to such hypothetical selective nucleases then the lack of expression in the yeast lysate could be explained in this way. This hypothesis was very simply tested by incubating a variety of ^{32}P - labelled messages in yeast and wheatgerm lysates and setting up the reaction conditions for active protein synthesis. Samples (10µl), were taken at different time - points, denatured, and loaded on a 1.5 % Agarose / Formaldehyde gel run for three hours and autoradiographed as in section 2:12:2. Any degradation of transcripts would be immediately apparent as a black smear on the autoradiograph. Labelled transcripts encoding ricin A chain, prepro - alpha factor, chicken lysozyme and preplastocyanin, (prepared from a cDNA donated by C.Robinson), were prepared as in section 2.9. Transcripts were incubated in a yeast and wheatgerm lysate under conditions for translation at 25°c and 29°c respectively. Samples were removed at 0, 30, 60, 90, and 120 minutes, then electrophoresed as described above. The dried gel was exposed to Fuji RX X - Ray film for 16 hours and the resultant autoradiographs are shown in figures 5:2:6. a.,b.,c. and d.

It is evident that there was little or no RNAase activity in either system whether the message encodes a protein of plant, animal or lower eukaryotic origin.

The conclusion can be drawn, therefore, that the lack of visible ricin A chain translation was not due to differential sensitivity of transcripts to nucleases. It should be remembered, also, that one of the components of a yeast lysate translation reaction is human placental ribonuclease inhibitor.

FIGURE 5.2.6. ANALYSIS OF RIBONUCLEASE ACTIVITY IN A YEAST LYSATE.

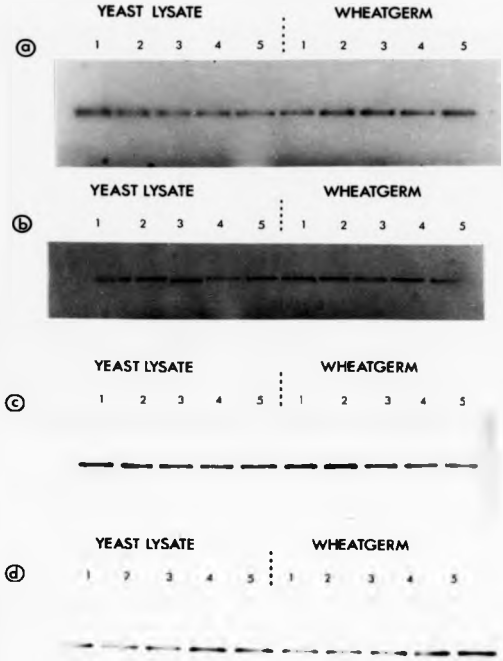
Transcripts encoding ricin A chain, prepro alpha factor and prepro plastocyanin were labelled and prepared as described in section 2:10 and incubated in a yeast and wheatgerm lysate at 25 and 29°c respectively for 1 hour as described in sections 2:11:3 and 2:11:1 respectively. Samples, (2ul), were removed at 0, 30, 60, 90 and 120 minute intervals (lanes 1-5 respectively), then analysed by electrophoresis on a 1.5% agarose formaldehyde gel as described in section 2:4:3.

Figure 5.2.6a = ricin A chain RNA

Figure 5.2.6b = prepro plastocyanin RNA

Figure 5.2.6c = chicken lysozyme RNA

Figure 5.2.6d = prepro alpha factor RNA.


YEAST LYSATE
WHEATGERM
a
1 2 3 4 5 1 2 3 4 5
YEAST LYSATE
WHEATGERM
b
1 2 3 4 5 1 2 3 4 5
YEAST LYSATE
WHEATGERM
c
1 2 3 4 5 1 2 3 4 5
YEAST LYSATE
WHEATGERM
d
1 2 3 4 5 1 2 3 4 5

Incorporation of ^{35}S - labelled methionine into TCA precipitable counts was also followed in the same reactions, 2µl samples being taken at the same time time points as the samples for RNA analysis and TCA precipitated as described previously. Figures 5:2:7., a., b., c. and d., are a graphical representation of the results. In all cases, except for ricin A chain in yeast lysate (figure 5:2:7.a.,), the same pattern is observed of the increase of TCA precipitable counts up to 30 - 60 minutes incubation followed by a plateau. No significant stimulation above background was observed in the yeast lysate when ricin A chain transcripts were added, in marked contrast to the other templates.

This simple series of experiments thus provided evidence to support several hypotheses;

1. Re - affirmation of the ability of plant messages to be translated in a yeast lysate system as in section 5:2:5:1.

2. Rejection of the hypothesis that differential translation is due to differential sensitivity of added transcripts to selective nucleases.

3. Although protein synthesis does not increase linearly over a two hour period this is not due to RNA degradation.

Lutcke and co - workers reported that SP6 - synthesised templates were stable in reticulocyte lysate systems for more than 8 hours and showed that a profile of protein synthesis in that system was much the same as observed in figure 5.2.7. (Lutcke et al., 1987).

5:2:5:4. Initiation of translation

The influence of the nucleotide at position -3 relative to the AUG codon on the initiation of protein synthesis has been studied in fine detail, (Lutcke et al., 1987). In the rabbit reticulocyte

FIGURE 5.2.7. ANALYSIS OF ^{35}S - METHIONINE INCORPORATION INTO TCA PRECIPITABLE PROTEIN DIRECTED BY FOUR DIFFERENT MESSAGES.

The incorporation of ^{35}S labelled methionine into TCA precipitable protein was followed in the same lysate samples as in figure 5.2.6. Samples of 2ul were taken at the same time points as samples for RNA analysis and TCA precipitated as described in section 2:11:1. Results are expressed graphically.

Figure 5.2.7a = ricin A chain

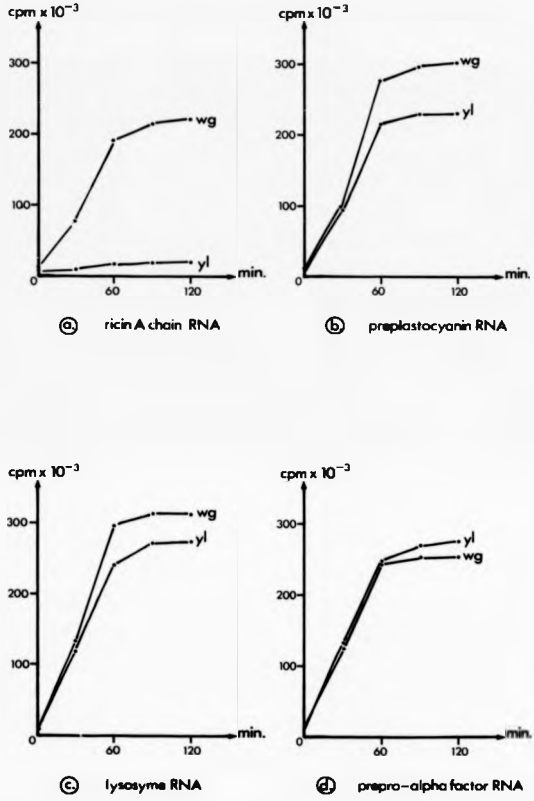
Figure 5.2.7b = prepro plastocyanin

Figure 5.2.7c = chicken lysozyme

Figure 5.2.7d = prepro alpha factor

wg = wheatgerm

yl = yeast lysate


cpm x 10⁻³
300
200
100
0
60
120
min.
wg
yl
ricin A chain RNA
preplastocyanin RNA
lysosyme RNA
prepro-alpha factor RNA

lysate studied, differences were noted in the level of translation between messages which only differed in the identity of the base at position -3. By the use of Oligonucleotide - directed mutagenesis, this base was changed from an A to C, U, or G. The initiation environment was found to have a profound effect on the level of translation. A hierarchy in the level of translation was observed of 100, 85, 61 and 38 %, for A, G, U and C, at position -3 respectively in the rabbit reticulocyte system but no effect on translation of the same message in a wheatgerm lysate. With such obvious similarities in these data to my observations on translational differences between the two systems , an examination of the sequences surrounding the AUG codons of the ricin cDNA's used was prompted. Sequences are:

ricin A chain	=	G G G G **A U G** T A
ricin B chain	=	A G C C **A U G** A A
proricin	=	G G G G **A U G** T A

If Lutcke's observations hold true for ricin transcripts then only a minor reduction in translational efficiency in the reticulocyte lysate should be observed compared to the wheatgerm system. Figure 5.2.3. indicated larger percentage differences in the level of translation between reticulocyte lysate and wheatgerm systems. Translation of ricin B chain in reticulocyte lysate was just 16 % of that in wheatgerm, for proricin, the level was 11 % , whilst that of ricin A chain was only 4 %. This was very different to the picture obtained by Lutcke and colleagues and that discussed by Kozak where some 205 messages out of atotal of 211 all had an A at position -3.

(Kozak, 1984). The abnormally large differences I have observed may be an artefact of the exceptionally efficient wheatgerm translation system used.

Lutcke went on to review the consensus sequences around the initiating codon of plant and animal messages. From a total of 61 plant messages, the consensus sequence was **A A C A A U G G C**, whilst from a total of 209 animal RNA sequences it was **C A C C A U G**.

Differences observed between wheatgerm and reticulocyte lysate translation of ricin encoding messages may thus be due partly to a poor initiation environment, (caused by the use of Xho 1 linkers immediately 5' to the AUG codon), in ricin A chain and proricin clones. Possibly, the relatively more efficient translation of ricin B chain with a ricin signal sequence in the rabbit reticulocyte lysate compared to ricin A chain and proricin may be a result of its slightly better initiation environment.

5:2:5:5. Polypeptide chain elongation / termination.

There are no reports in the literature of inefficient polypeptide chain elongation or termination by cell - free translation systems. Indeed one of the requirements of such systems is that not only should they respond to exogenous mRNA addition by initiating protein synthesis, but that they should also ensure efficient elongation and termination. This was therefore not considered to be a likely cause for differential expression of ricin A chain.

5:2:5:6. Protein degradation.

The yeast lysate prepared for use in this study was derived from a protease deficient strain of Saccharomyces crevisiae. ABYS 1. This is a quadruple mutant lacking the four vacuolar peptidases;

proteinase A, proteinase B, carboxypeptidase Y and carboxypeptidase S. Even in this strain, additional proteases have been identified, (Achstetter et al., 1984).

Section 5.2.5.3. presented data which showed:

1. The cessation of de novo protein synthesis after 30 - 60 minutes in wheatgerm and yeast lysates , (see figure 5.2.7.), was not due to RNA degradation.

2. Over a two hour translation period protein accumulation did not increase linearly. For some of the proteins expressed, (lysozyme and prepro - alpha factor: figure 5.2.7c and d.), protein accumulation continued longer than for others (preplastocyanin: figure (5.2.7d.). This is particularly noticeable in the yeast lysate translations.

3. Ricin A chain accumulated to a significant level in the wheatgerm system but not at all in the yeast lysate.

Thus, if RNA degradation is not responsible for these phenomena, then it is possible that selective proteolysis is occuring, in cases where the accumulation of a protein is not as extensive as for others eg., preplastocyanin vs. lysozyme, or in extreme cases, eg., ricin A chain, where the protein is destroyed as soon as it is made. If ricin A chain were highly sensitive to proteases present in a yeast lysate, its accumulation would be prevented and it would thereby remain undetected.

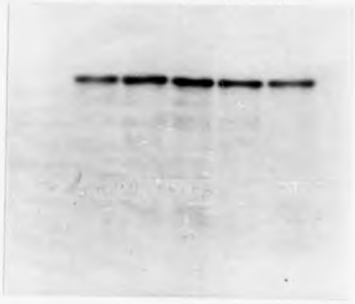
This was very easy to test experimentally. All of the messages available translated efficiently in a wheatgerm lysate. (See section 5.2.1.). Lysozyme, prepro - alpha factor and ricin A chain RNA's weretranslated for one hour in a wheatgerm lysate in the presence of ^{35}S - labelled methionine. A 2µl sample of each reaction was added to a yeast lysate system set up for translation at 25°c. At 0, 15, 30,

FIGURE 5.2.8. PROTEASE ACTIVITY IN YEAST LYSATE.

Chicken lysozyme, prepro alpha factor and ricin A chain were translated for 1 hour in a wheatgerm lysate and 2µl of this was added to a yeast lysate set up for translation at 25°c. At 0, 15, 30, 45 and 60 minute intervals, (lanes 1 to 5 respectively), 2µl aliquots of each reaction were removed and analysed by SDS - Polyacrylamide gel electrtophoresis, fluorography and autoradiography as described in section 2:12:3.

RICIN A CHAIN

1 2 3 4 5

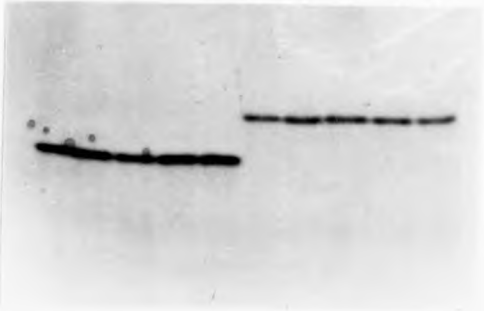


CHICKEN LYSOSYME

1 2 3 4 5

PREPRO ALPHA-FACTOR

1 2 3 4 5



45 and 65 minutes, 2ul aliquots were removed and analysed by SDS - PAGE, fluorography and autoradiography of the dried gel.

Results are presented in figure 5.2.8.

There is no evidence , (at this level of stringency at least,), that proteolysis is a significant factor in the yeast lysate in preventing the accumulation of ricin A chain to a level detectable by autoradiography. Indeed, ricin A chain is known to be moderately resistant to many proteases These data show it to be resistant to the proteases identified by Achstetter and colleagues in the protease deficient yeast strain, ABYS 1. (Achstetter et al., 1984). Only the yeast lysate was tested in this manner for the presence of proteases, since the absence of ricin A chain detectable by autoradiography of fluorographed translation products was so marked in this system.

5.2.5.7. Toxicity of the protein synthesised.

It is possible that some property of the protein expressed could in some way affect its subsequent accumulation by interfering with the translation "machinery" of the system. The toxic activity of ricin A chain or lectin activity of ricin B chain could be causing such traumas. An explanation as to why translation of proricin in a cell - free system should cause ribosome inactivation, thereby preventing its subsequent translation and accumulation is proposed at a later stage. Since ricin A chain was not detected by autoradiography of SDS - PAGE samples of a yeast lysate translation it must exhibit extreme potency in order to inactivate the ribosomes whilst accumulating to levels below this threshold of detection. For this to be so it is necessary to propose that:

1. Ricin A chain translated in vitro is biologically active.

2. Differences in accumulation of ricin A chain between the three cell - free systems are a result of differential sensitivity of ribosomes in each system to ricin A chain. The differential sensitivity of plant and animal ribosomes to ricin has been well documented (Cawley et al., 1977, Cawley et al., 1979, Olsnes and Pihl, 1982.). It is estimated that native ricin A chain is 100 - 1000 times more active on mammalian than on wheatgerm ribosomes.

The toxicity of ricin A chain in vitro is explored in detail in the next chapter, however the damaging consequences of ricin B chain lectin activity are less easy to investigate. For the assay of ricin B chain produced from SP6 transcripts, the accumulation of significant amounts of it are essential. For this reason the Xenopus oocyte in vivo translation system was chosen by Richardson and colleagues for the analysis of recombinant ricin B chain. (Richardson et al., 1988). A functional assay for in vitro synthesised ricin B chain would not be feasible. P. Richardson has investigated the expression of ricin B chain in yeast in some detail, (Richardson et al., 1988b). It was found that ricin B chain expressed cytoplasmically without an N - terminal signal peptide accumulated to significant levels but was insoluble. Ricin B chain expressed from a cDNA fused at its 5' end with the signal sequence of prepro - alpha factor or that of preproricin was targetted to vacuoles and was biologically active as determined by an in vitro assay.(P. Richardson, personal communication.).

Since in vivo expression of ricin B chain in yeast was tolerated by the host, it must be assumed that the inability of yeast lysates to accumulate expressed ricin B chain to a level detectable by autoradiography was not due to the activity of ricin B chain expressed at low levels.

SECTION 5:3. DISCUSSION

The transcription of a ricin A chain cDNA sequence cloned downstream from a T7 promoter, (section 3.3.2.) was directed efficiently by T7 RNA polymerase in vitro. (chapter 4). Transcripts were accurately and efficiently translated in a wheatgerm and rabbit reticulocyte but not in a yeast lysate. Marked differences were observed in the level of accumulation of 35S - labelled ricin A chain polypeptide between the three systems. (figure 5.2.3.). Other messages encoding prepro - alpha factor, chicken lysomyme and preplastocyanin were translated efficiently in all three systems. The differential accumulation of ricin A chain was most marked between that observed in the wheatgerm lysate and the yeast lysate in which it could not be detected by autoradiography. This difference was investigated experimentally by examining RNA degradation and protease activity in the lysates under optimal working conditions.

It was shown conclusively that differences were not due to selective ribonuclease activity in two separate experiments. The first, (see figure 5.2.5.a. and b., lane 8), showed that both ricin A chain and prepro - alpha factor transcripts were not degraded at the end of 1 hours translation and that the same batch of transcripts could nevertheless be translated in a wheatgerm system. (see figure 5.2.5.a and b., lane 9). In the second set of experiments RNA degradation was examined in more detail. Radiolabelled messages encoding plant, animal and lower eukaryote proteins were incubated in a yeast lysate under optimal conditions. Even after two hours , the RNA was still intact, supporting the observations of Lutcke and colleagues, (1987), discussed in section 5:2:5:4. The conclusions drawn from section

5:2:5:4. were that the poor initiation environment of the ricin constructs could be in part, a cause of the variation in translational efficiency seen between the three cell - free systems. This form of translational control has received much attention in the literature. (Kozak, 1983, Kozak, 1984, Chen and Strubl, 1985, Hamilton, 1987,). A definite consensus sequence has been identified surrounding the initiation codon of plant messages. The sequence described by Lutcke and colleagues from a survey of 61 plant messages was;

A A C A **A U G** G C . The sequence surrounding the codons designated to initiate translation of ricin A chain and proricin cDNA's was;

G G G G **A U G** T A . The mutagenesis studies of Lutcke and co - workers, (1987),in vitro. and those analysed in transfected COS cells by Kozak, (Kozak, 1984b. and 1986,) and in yeast by Sherman and co - workers, (Baim et al., 1985), have indicated that nucleotide -3 modulates rates of protein synthesis. The effects in yeast were less, (2 - 3 fold), than those observed in animal cells, (4 - 20 fold).

The most significant conclusion that was drawn was that in a wheatgerm system the identity of nucleotide -3 had no effect on translation whereas any nucleotide other than an adenosine at -3 had a profound effect on reticulocyte lysate translation. A yeast lysate was not studied in this work. Since this is much the same pattern as I have observed and since all ricin constructs have a poor initiation environment, having a G at position -3, then this may have the effect of reducing the amount of translation in a rabbit reticulocyte lysate compared to the wheatgerm.

Differential protease sensitivity was conclusively rejected as being a cause of differential accumulation of protein. Ricin A chain

synthesised in a wheatgerm remained intact after two hours incubation in a yeast lysate set up for active translation.

This suggested differential accumulation of ricin A chain was due to the expression of functional ricin A chain displaying ribosome inactivating activity. Differences in its accumulation could be attributed to differential sensitivity of ribosomes from plant, animal and lower eukaryote sources. The greater their sensitivity, the lower the level of accumulation of ricin A chain supported by the system expressing it. Thus in a wheatgerm, with relatively insensitive ribosomes accumulation is observed, less in the rabbit reticulocyte lysate, with ribosomes 100 - 1000 times more sensitive, (Cawley et al., 1977, Cawley et al., 1979), and even less in a yeast lysate.

CHAPTER 6.

THE DESIGN OF A SENSITIVE METHOD TO ASSESS THE BIOLOGICAL ACTIVITY OF CLONED RICIN A CHAIN *in vitro*.

SECTION 6:1 INTRODUCTION

It was suggested at the end of the previous chapter that ricin A chain expressed in vitro from SP6 / T7 transcripts was biologically active.

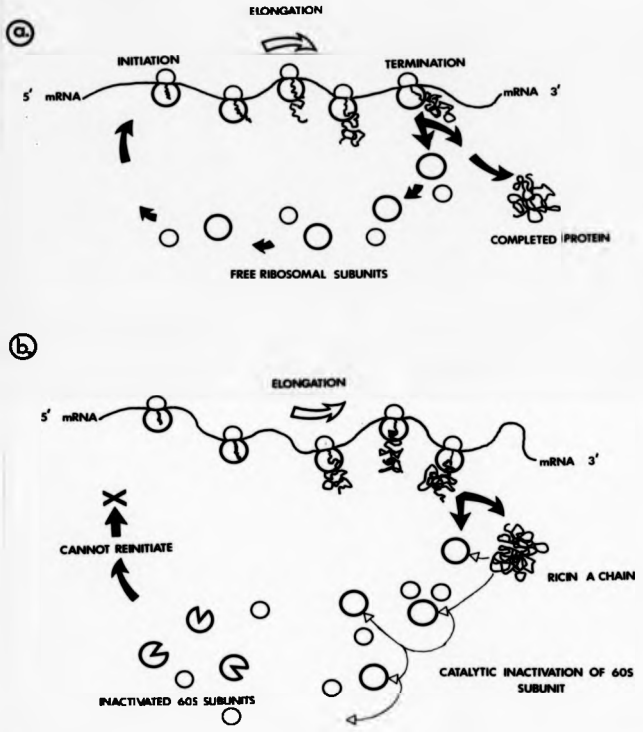
At the outset of this work, there were few reports in the literature indicating that the in vitro synthesis of biologically active protiens in an RNA - dependent cell - free system was possible. Subsequently, Glass and colleagues successfully synthesised biologically active ornithine decarboxylase (ODC), in a rabbit reticulocyte lysate, (Glass et al., 1987). This was a particularly significant achievement since ODC has a biological half - life identified as being one of the shortest of any mammalian protein. This approach enabled the production of sufficient quantities of easily obtainable ODC for a detailed analysis of its function for the first time.

For the biological activity of a protein expressed in an RNA - dependent cell - free system to be assessed rapidly, two requirements concerning the protein would need to be fulfilled. 1. The protein should be expressed in sufficient quantities for assay to be possible without the need for purification from the lysate.

2. If the protein is not expressed in large quantities, then its specific activity should be of sufficient magnitude to allow its assay at low levels of expression.

Ricin A chain is a catalytic inhibitor of eukaryotic protein synthesis of extreme potency. Even though its accumulation in rabbit reticulocyte and yeast lysates is not as great as that noted for other proteins in this study, its extreme potency, stability and the catalytic, irreversible nature of its activity would support the contention that it was expressed in an active conformation. Indeed, an enzyme with such a high specific activity and posessing the ability to irreversibly inactivate the system synthesising it would not be expected to accumulate to any extent. Differential expression between lysate systems could be explained in terms of differential sensitivity of ribosomes in that system to expressed, biologically active ricin A chain. In data presented in section 5.2, the greater accumulation of ricin A chain in a wheatgerm compared to a reticulocyte lysate could be explained on the basis that the wheatgerm ribosomes, being less sensitive to ricin A chain, allow its more substantial accumulation than is supported in the rabbit reticulocyte system. This hypothesis is summarised schematically in figure 6.1. Figure 6.1a. shows the response of ribosomes to the translation of a protein which does not damage them such as prepro - alpha factor, chicken lysozyme or preplastocyanin. Subsequent translation is made possible by a continuous recycling of intact ribosomal subunits. Also, where the ribosomes of a system are resistant to ricin A chain attack, they remain unaffected and can recycle, allowing a substantial accumulation of the protein, as in the case of ricin A chain translation in a wheatgerm lysate. In contrast, figure 6.1b. depicts a system, the ribosomes of which, are sensitive to ricin A chain attack, such as a

Figure 6. 1.


a
ELONGATION
INITIATION
TERMINATION
5' mRNA
mRNA 3'
COMPLETED PROTEIN
FREE RIBOSOMAL SUBUNITS
b
ELONGATION
5' mRNA
mRNA 3'
CANNOT REINITIATE
RICIN A CHAIN
CATALYTIC INACTIVATION OF 60S SUBUNIT
INACTIVATED 60S SUBUNITS

rabbit reticulocyte lysate. The translation of ricin A chain T7 template was directed by ribosomes, which, when the completed protein is released, starts to catalytically and irreversibly inactivate them. The extreme potency of ricin A chain, coupled with its catalytic activity mean that it rapidly depletes the pool of ribosomes rendering them incapable of recycling and thus supporting subsequent translation. Furthermore, the amount of ricin A chain necessary to inactivate the system is low enough to allow it to escape detection by autoradiography. Just how fast this occurs would depend on the pool size and sensitivity of ribosomes in that system to ricin A chain attack. The more sensitive they are, the more rapidly the pool of ribosomes is depleted and the lower the level of accumulation of ricin A chain seen. If this hypothesis is correct one could predict yeast ribosomes to be more sensitive than rabbit reticulocyte ribosomes.

An experiment was proposed in order to substantiate this hypothesis as follows;

If biologically active ricin A chain is synthesised in a cell - free system and if the ribosomes of that system are sensitive to its catalytic activity then they should be inactivated. The inactivation of ribosomes was assessed by adding a second message which, under ideal conditions should be actively expressed. Full details of this experiment are rigorously examined in section 6:2.

SECTION 6:2 RESULTS.

6:2:1 Inactivation of rabbit reticulocyte ribosomes by ricin A chain expression.

From results discussed in chapter 5, it was evident that de novo translation of a message added to a rabbit reticulocyte lysate was not initiated after 30 minutes incubation. If ribosome inactivation is to be assessed in terms of the inability of the system to support translation of a second message added after a period of translation, then it was essential to establish two very important criteria;

1. The minimum length of time required for the accumulation of sufficient ricin A chain to completely inactivate the ribosomes, but to allow the translation of a second message if the first message encodes a non - ribosome inactivating protein.

2. The maximum length of time for which a lysate can be incubated before it becomes incapable of supporting the efficient translation of a second message.

The approach taken was to allow ribosomes which are very sensitive to ricin A chain, namely, rabbit reticulocyte ribosomes, to translate mRNA encoding recombinant A chain. Assuming that the A chain folds into a catalytically active conformation, the newly synthesised toxin should immediately begin to inactivate ribosomes and should rapidly render them incapable of translating a second transcript added subsequently. When the ribosomes have been translating in vitro

synthesised transcipts encoding non toxic yeast preproalpha factor or non-toxic ricin B chain, they should synthesise both polypeptides. Using the latter translation as a control, one has the basis of a valid assessment of the biological activity of expressed protein. Otherwise, one could argue that the inability of the system to support subsequent translation is not due to ribosome inactivation but due instead to depletion of vital translation components, or some other breakdown of the system. One relies heavily on the efficient translation of the control messages for the valid interpretation of data.

An initial experiment was set up to substantiate these criteria, the results of which are shown in figure 6.2.1. 1µl of ricin A chain message, chicken lysosyme message or no message were translated for one hour in a rabbit reticulocyte lysate as described in section 2:11:2. 1µl of either ricin A chain or lysosyme message was then added to each translation for a further hour, then 5ul samples electrophoresed on a 10% SDS - Polyacrylamide gel, fluorographed and autoradiographed as described in section 2:12:3.

Two important conclusions can be drawn from the results;

1. Efficient translation of a message added after one hours incubation was initiated, (lysosyme, Lane 6.).

2. The translation of ricin A chain RNA for the first hour completely abolished the translation of lysosyme message in the second hour, (Lane 2.).

This evidence was encouraging but not without scope for criticism. Two main obvious criticisms of these data were;

FIGURE 6.2.1. A PRELIMINARY INVESTIGATION INTO THE EFFECT OF RICIN A CHAIN TRANSLATION ON SUBSEQUENT TRANSLATION IN A RABBIT RETICULOCYTE LYSATE.

The first message was translated for 1 hour at 37°c in a rabbit reticulocyte lysate as described in section 2:11:2, then a second message (1µl) added for a further hours incubation. At the end of this time, 5µl of each translation was loaded onto a 10% SDS - Polyacrylamide gel, run at 16mA for 12 hours, fluorographed and autoradiographed as described in section 2:12:3:1.

Samples of RNA were added for the times detailed above, in the order given below:

Lane 1 = ricin A chain then ricin A chain

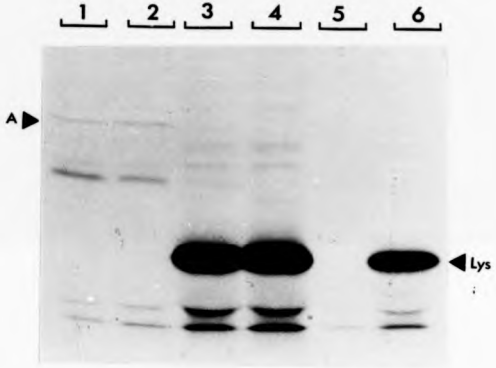
Lane 2 = ricin A chain then chicken lysozyme

Lane 3 = chicken lysozyme then ricin A chain

Lane 4 = chicken lysozyme then chicken lysozyme

Lane 5 = no RNA then ricin A chain

Lane 6 = no RNA then chicken lysozyme.


1
2
3
4
5
6
A
Lys

1. As a system for the rapid assessment of the activity of ribosome inactivating mRNAs, two hours was a rather long time.

2. Although de novo initiation of translation in a rabbit reticulocyte lysate had been proven after a period of one hour had elapsed, (Lane 6), it had not been proven that in a system where active translation had been occurring for the first hour, that the system was capable of supporting translation during the second hour. In lane 2, chicken lysosyme synthesised in the first hour cannot be distinguished from that synthesised during the second.

Three improvements were made in a second, more extensive investigation;

1. The first message was translated for one hour but the second was translated for only half an hour to shorten the reaction time.

2. In the second half hour a different, highly expressed protein - encoding message was added, to that used in the control reaction.

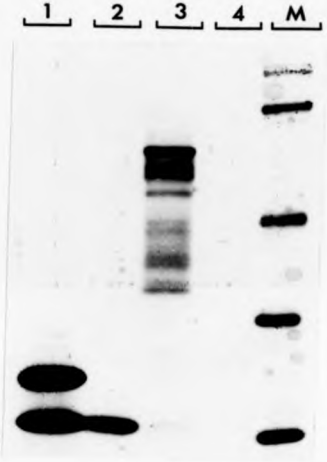
3. The toxicity of ricin B chain and proricin was analysed. This system offered the unique opportunity to investigate the toxicity of these proteins away from even trace amounts of ricin A chain which would contaminate B chain or proricin purified from plant tissue.

Ricin A chain message, ricin B chain message, proricin message and prepro - alpha factor message were translated for one hour then 1ul of chicken lysosyme message was added and the reactions incubated for a further half - hour. Samples, (5µl), were run on a 10% SDS - Polyacrylamide gel, fluorographed and autoradiographed as described in section 2:12:3. Results are shown in figure 6.2.2.

FIGURE 6.2.2. THE ABILITY OF A RABBIT RETICULOCYTE LYSATE TO SUPPORT TRANSLATION SUBSEQUENTLY TO RICIN A CHAIN, RICIN B CHAIN, PRORICIN, OR PREPRO ALPHA FACTOR TRANSLATION.

1ul of prepro alpha factor RNA, (lane 1), ricin B chain RNA, (lane 2), proricin RNA, (lane 3), or ricin A chain RNA, (lane 5), was translated in a rabbit reticulocyte lysate as described in section 2:11:2 for 1 hour. 1µl of chicken lysozyme RNA was then added to each translation for a further 30 minutes, then 5µl samples were electrophoresed on a 10% SDS - Polyacrylamide gel, fluorographed and autoradiographed as described in section 2:12. Lane M represents ^{14}C labelled molecular weight markers.

Ricin B chain polypeptide is absent in this translation. It does, however, not impair the subsequent translation of a second message as shown in figure 7.2.2.


1
2
3
4
M

Further evidence was provided by this very simple approach, to substantiate the hypothesis that ricin A chain was being expressed with a specific activity of sufficient magnitude to totally inactivate rabbit reticulocyte ribosomes, (Lane 4.). Furthermore, even though very efficient translation of prepro - alpha factor occurred in the first hour, the efficient translation of a second message was possible subsequently, (chicken lysozyme, lane 1.). One observation of this experiment was that proricin showed partial ribosome inactivating activity, (Lane 3.) as demonstrated by a trace of lysozyme production.

Many reports, eg. Olsnes and Pihl, (1982), indicated that non - reduced, heterodimeric ricin is catalytically inactive. Reduction of the interchain disulphide bond is necessary to liberate the A chain for it to display full catalytic activity presumably because in the heterotoxin, the active site on ricin A chain is stearically blocked by the associated B chain.

During ricin biosynthesis in Ricinus seeds, the A and B chains are initially synthesised as part of a single precursor polypeptide (Butterworth and Lord, 1983), where the two polypeptides of the mature toxin are covalently linked together by a 12 amino acid linker or joining peptide. In the plant cell, proricin is transported from the ER, via the golgi apparatus, to the protein bodies, where a specific acid endoprotease removes the linker peptide to liberate the A and B chains.(Lord, 1985 and 1985a). Since release of ricin A chain from the heterodimeric holotoxin is necessary for it to become catalytically active, it has always been assumed that the A chain

component of proricin would likewise be inactive. The translation of proricin from pSP64T allows a unique opportunity to investigate this question. Proricin translated from pSP64T in a cell - free system still contains the linker region. Therefore for proricin to display ribosome inactivating activity in vitro, there are two possibilities:

1. Proricin was toxic, but not as toxic as ricin A chain.

2. Cleavage and reduction of proricin occured after it was produced, so that as the reaction continued, dissociated subunits were released and accumulated and ribosome inactivation occured.

Evidence to support the latter possibility was provided recently by M. Westby and P. Richardson, (this lab publication in press).

Proricin was translated in Xenopus oocytes after injection of pSP64T AB RNA. The protein was isolated, reduced and run on a Sepharose G75 column. The catalytic activity of fractions collected from this column was estimated by assessing the N - glycosidase activity of each fraction. This was achieved by adding aliquots of each fraction to duplicate, purified rabbit reticulocyte ribosomes. Ribosomal RNA was extracted from each sample and then treated with or without aniline as described by Endo et al., (1987), and run on a formamide gel as described in section 2:4:4. The release of a 460 base pair fragment was indicative of ricin A chain activity. No N - glycosidase activity was noted for the 60kD proricin fraction, but activity was noted for a 30kD fraction, probably representing ricin A chain produced by the cleavage of the linker peptide and inter chain disulphide bond. For this to be possible, one must postulate the

existence of enzymes capable of catalysing this cleavage in the Xenopus oocyte.

By drawing a comparison between an in vivo and an in vitro system in suggesting enzymatic cleavage in the latter, one is assuming that the enzyme present in the in vivo system is present in vitro. However, despite misgivings about making such a large assumption, it seems probable that such an event is in fact occuring in vitro. Proricin, synthesised from the same template in both systems was proven conclusively to show no N - glycosidase activity in vivo. Partial toxicity could be explained in light of this work. For proricin to accumulate to the levels it did and to be continuously in contact with sensitive ribosomes in ever increasing concentrations, it must have very low toxicity compared with ricin A chain to allow trace lysozyme synthesis. Furthermore, if it were toxic, one would not expect its accumulation to the extent seen. What seems more likely in the light of the evidence above is that there was a slow release of ricin A chain after enzymic cleavage of the linker region, the amount of ricin A chain released will determine how many ribosomes remain active and capable of lysozyme translation subsequently. The amount of ricin A chain released and therefore the amount of ribosome inactivation which will occur will depend on several factors:

1. The presence of an enzyme capable of cleaving the linker peptide joining the ricin A and B chains in vitro.

2. If this enzyme exists, the amount of peptide cleavage will depend on; a) the amount of proricin synthesised,

b) conditions in vitro being suitable for its activity

c) the half life of the enzyme.

One would predict a lag period, before ricin A chain starts to accumulate and ribosome inactivation would occur, the length of which would depend on the above factors. If, by the time lysozyme RNA was added, there were ribosomes still active, one would envisage the synthesis of lysozyme, the amount of lysozyme produced being dependent on the duration of the lag period. One could predict that as a result of trace lysozyme synthesis, that few ribosomes were left intact and that the lag period was not long. All of this is only one interpretation of the data, but it is an entirely feasible conjecture within the limitations of the experimental approach, since proricin is clearly only partially cleaved. Reports also indicate that as the two ricin subunits are released, the A chain adopts an active conformation and that when linked to the B chain it is inactive, presumably because the active site of ricin A chain is sterically blocked.

One very great assumption made initially in this argument was that the N - glycosidase activity of ricin A chain is a true reflection of its catalytic activity. In other words, is the release of Adenine 4324 from 28s RNA the cause of inhibition of translation and irreversible ribosome inactivation or are they merely two unlinked phenomena ? To date, no one has attempted to investigate whether these two properties of ricin A chain are functionally linked. However, recently, Moller and colleagues have presented data which strongly suggest that depurination at this specific site prevents EF 1 and EF 2 binding to 28S rRNA and therefore **directly** inhibits protein synthesis.

(Moazed et al., 1988). For the N - glycosidase activity of ricin A chain to be used as a diagnostic assay of its activity, it is essential to establish this relationship. Section 6:2:3 attempts to investigate this in detail.

6:2:2. Wheatgerm ribosomes are not inactivated by ricin A chain.

The same experiment as in section 6:2:1 was conducted in a wheatgerm lysate to explore the sensitivity of wheatgerm ribosomes to ricin A chain expression. Ricin A chain RNA, (1µl) was translated for one hour in a wheatgerm lysate at 29° c. At the end of this time, 1ul of prepro - alpha factor RNA was added and the reaction continued for a further hour. In a separate reaction, no RNA was incubated for one hour, followed by prepro - alpha factor RNA for a further hour. Samples, (5ul) of the translation mixtures were run on a 10% SDS - polyacrylamide gel and an autoradiograph of the fluorographed gel is shown in figure 6.2.3. It is evident, by a comparison of lanes 2 and 3, that the translation of ricin A chain for the first hour does not inhibit the translation of prepro - alpha factor RNA during the second hour.

FIGURE 6.2.3. **ANALYSIS OF THE EFFECT OF RICIN A CHAIN TRANSLATION ON SUBSEQUENT TRANSLATION IN A WHEATGERM LYSATE.**

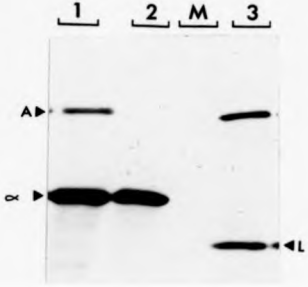
The first message, (1µl), was translated for 1 hour at 28°c in a wheatgerm lysate as described in section 2:11:1, then a second message, (1µl), was added for a further hour. At the end of this time, 5µl of each reaction was loaded on a 10% SDS - Polyacrylamide gel, run at 16mA for 12 hours, fluorographed and autoradiographed as described in section 2:12:3:1. Samples were loaded in the following order:

Lane 1 = ricin A chain then prepro alpha factor

Lane 2 = no RNA then prepro alpha factor

M = ^{14}C labelled molecular weight markers

Lane 3 = ricin A chain then chicken lysozyme


1
2
M
3
A
α
L

6.2.3. The relationship between inhibition of protein synthesis and N [illegible] activity of ricin A chain.

Until recently, the molecular mechanism of action of ricin and other related ribosome inactivating proteins had not been elucidated. The fungal toxin, sarcin, was known to hydrolyse a single phosphodiester bond between G_{4325} and A_{4326} in an evolutionally conserved region of 28s rRNA, (Chan et al., 1983). No corresponding endonuclease or ribonuclease activity was associated with ricin, (Mitchell et al., 1976). Endo and colleagues showed that treatment of rat liver ribosomes with ricin resulted in the removal of a single base (A_{4324}), in rat 28s rRNA whilst leaving the sugar phosphate backbone intact (Endo et al., 1987, Endo and Tsurugi, 1987). The phosphodiester bonds on either side then became susceptible to aniline catalysed hydrolysis in vitro. resulting in the release of a 460 base pair fragment.

Although it is now accepted that ricin A chain causes the removal of an adenine from a highly conserved loop in the rRNA of eukaryotic 60s subunits, it is not known whether this modification in itself is sufficient to inhibit protein synthesis, however, the work of Moller and colleagues would certainly suggest a causal relationship. (Moazed et al., 1988). The N - glycosidase activity of ricin is extremely quick and simple to demonstrate and has been shown in this lab to be dose - dependent. It is therefore a very attractive method for the diagnostic detection of ricin activity. One obvious application would be in the confirmation of cell killing by specifically targeted

ricin (eg as an immunotoxin), in cell types where the the analysis of inhibition of translation is hard or impossible to detect or for the detection of immunotoxin penetration at various depths into a tumour, where to date no suitable method of analysis is available. Work currently in progress in this lab to study the expression of ricin A chain in yeast relies heavily on this approach. Upon induction of ricin A chain expression, cell death is rapid. However, the presence of ricin A chain is not detected by immunoprecipitation or western blotting. It was suspected that ricin A chain was being expressed and was the cause of cell - death yet was accumulating to levels well below the level of resolution employed. By the treatment of ribosmal RNA isolated from yeast induced to express ricin A chain with aniline it was shown that depurination of the RNA had indeed occured indicating that ricin A chain had been synthesised, albeit at a very low level.

For these ambitions to be realised, however, it remains essential to link the two activities of ricin functionally. To this end, a series of experiments were undertaken by Dr. Martin Hartley and myself.

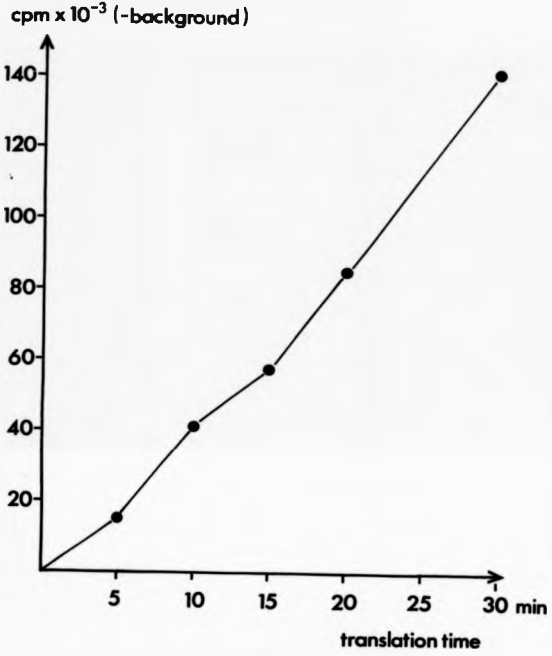
The yeast lysate system was chosen for this work for two main reasons:

1. RNA added to a yeast lysate system is highly expressed under normal conditions, (see figure 6.2.4.), but when ricin A chain is added translation is abolished in a dose - dependent manner. (data not shown).

2. It has been confirmed in this lab that the site of action of ricin A chain on 26s rRNA isolated from a yeast lysate is at exactly

FIGURE 6.2.4. TRANSLATION OF WHEAT POLY A$^+$ RNA IN A YEAST LYSATE: INCORPORATION OF ^{35}S METHIONINE INTO TCA PRECIPITABLE PRODUCTS

1µl of wheat Poly A$^+$ RNA was translated in a yeast lysate for 30 minutes as described in section 2:11:3. At time points of 5, 10, 15, 20 and 30 minutes, 2µl aliquots were removed, in duplicate, spotted onto Whatman No. 1 discs and TCA precipitated as described in section 2:11:1. Discs were counted in 4ml of Beckman EP scintillant on an LKB 1212 minibeta scintillation counter. The means of counts obtained were plotted against time.


cpm x 10⁻³ (-background)
140
120
100
80
60
40
20
5
10
15
20
25
30 min
translation time

the same position in the conserved loop identified by Endo and colleagues.

Furthermore, the sequence and secondary structure of yeast 26S rRNA is known (Veldman et al., 1981).

The aim of the work described below was to link these two as yet, isolated observations, functionally.

Five separate 50µl yeast lysate translations were set up as described in section 2:10:3. The reactions varied in the amount of ricin A chain added to them in a concentration range of 200, 20, 2, 0.2 and 0ng. The reactions were preincubated with ricin A chain for 20 min and translation started by the addition of ^{35}S methionine at t=20. Translation was carried out for 10 min and duplicate 2µl samples were removed at 0, 1, 5 and 10 min., spotted onto 1cm Whatman M^{cr}.1 discs and TCA precipitated as described in section 2:10:1. From the remainder of the reaction mix, rRNA was extracted as described by Endo et al., 1987 and run on a formamide gel as described in section 2:4:4. The gels were stained in EtBr and scanned using a Joyce Lobell Chromoscan. The size of the fragment released by subsequent aniline cleavage was calculated to be 369bp, from the sequence of yeast 26S RNA described by Veldman et al., (1981). The interpretation of the data posed several problems, largely due to the variable base line of the scan. Several approaches were adopted to determine a value for the percentage modification of yeast 26S rRNA by ricin A chain.

1. If it is assumed that the 369bp fragment is derived from the 26S rRNA, then the areas under the peaks on the scan printout for the

fragment and 26S rRNA could be calculated and the former expressed as a percentage of the latter to give a value of the percentage modification of the RNA. However, due to the presence of minor breakdown products and a variable base line, this would lead to cumulative inaccuracies, particularly where minor modification at low toxin concentrations occurred.

2. A more accurate method was to determine the molar ratios of the fragment compared to a standard. Since the 16S rRNA peak does not change, whatever ricin concentration was used, this would be a suitable standard of length 1798 nucleotides, (Veldman et al., 1981). The height of the fragment peak and the 16S rRNA peak was measured:

Ricin A chain conc".	Height of 16S peak	Height of fragment peak
200ng/50µl	75mm	31mm
20ng/50µl	78mm	31mm
2ng/50µl	78mm	17mm
0.2ng/50µl	76mm	6mm
0.0ng/50µl	62.5mm	-

Ratio of 16S / fragment = 1798 / 369 = 4.886

Molar ratio = height of fragment x 4.886 / height of 16S

Ricin A chain conc".	Molar ratio
200ng	2.02
20ng	1.94
2ng	1.07
0.2ng	0.39
0ng	-

The percentage inhibition of protein synthesis was calculated as follows:

Ricin conc^n.	mean cpm t=20(a)	t=30(b)	b-a=c	$100 - \left[\frac{c}{19886 \times 100}\right]$
200ng	4022	6227	2205	89%
20ng	4359	6324	1965	90%
2ng	3869	8702	4833	76%
0.2ng	4377	15236	10859	45%
0ng	4942	24828	19886	0%

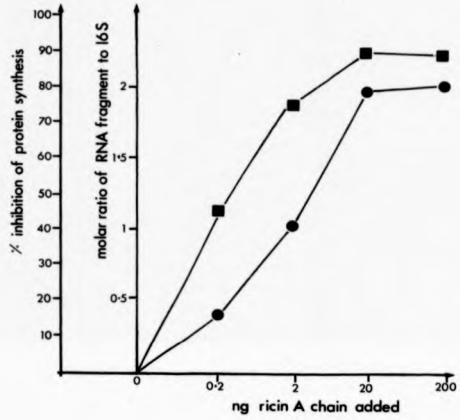
Thus the relationship of inhibition of translation to modification of the ribosomal RNA is:

Ricin conc^n.	Molar ratio of rRNAs	%age inhibition of protein synthesis
200ng	2.02	89%
20ng	1.94	90%
2ng	1.07	76%
0.2ng	0.39	45%

The percentage inhibition of translation and modification are both plotted against ricin A chain concentration in figure 6.2.5. From a comparison of the shape of the two curves it is evident that both the percentage inhibition of protein synthesis and the proportion of the ribosomal RNA modified show similar responses to ricin A chain action. At lower ricin concentrations greater inhibition of protein synthesis

FIGURE 6.2.5 THE RELATIONSHIP BETWEEN THE INHIBITION OF PROTEIN SYNTHESIS AND MODIFICATION OF 28S rRNA BY RICIN A CHAIN

The calculated values for the percentage inhibition of protein synthesis and modification of ribosomal RNA expressed as the molar ratio of fragment : 18S rRNA were plotted against the concentration of ricin A chain added to each of the 5 translations.


% inhibition of protein synthesis
100
90
80
70
60
50
40
30
20
10
molar ratio of RNA fragment to 16S
2
1·5
1
0·5
0
0·2
2
20
200
ng ricin A chain added

is observed than modification of the RNA. However this may be due to a greater inaccuracy in measurement of the smaller peak. Without conducting more elaborate experiments, little more can be said, other than the two functions of ricin A chain action seem to show an approximately empirical relationship.

SECTION 6:3. DISCUSSION.

Data presented in this chapter justify the contention that differences in the expression of ricin A chain run - off transcripts seen in the three cell - free systems discussed in chapter 5, are due to the toxicity of the translated A chain. Three conclusions were drawn from this work.

1. When recombinant ricin A chain transcripts were translated in a rabbit reticulocyte lysate, the ribosomes were rapidly inactivated as shown by their inability to support translation of yeast prepro - alpha factor, or chicken lysosyme encoding transcripts added subsequently. In contrast, ribosomes which have translated transcripts encoding non-toxic polypeptides such as ricin B chain, translate the second message under identical conditions.

2. Ribosome inactivation is accompanied by a highly specific modification to 28S RNA which occurs at the same position as that reported by Endo and colleagues, (1987). An attempt was made to correlate the N - glycosidic activity of ricin A chain with its ability to inhibit translation, in an effort to establish whether the former is

the cause of the latter. This question is explored in detail in chapter 11.

3. Protein synthesis by wheatgerm ribosomes was not inhibited under the conditions which inhibit rabbit reticulocyte ribosomes. This confirmed earlier observations that plant ribosomes are much less sensitive to inhibition by ricin A chain than are mammalian ribosomes.

A simple, sensitive approach was thus developed in which the expression and assessment of biological activity, (inhibition of protein synthesis), were combined. This was feasible because ricin A chain is an extremely potent inhibitor of mammalian ribosomes, (K_{cat} for ribosomes of 1500 / min., Olsnes et al., 1975,). It has been reported that a single A chain generated cytoplasmically is sufficient to promote the death of a cell given sufficient time. When rabbit reticulocyte ribosomes translated ricin A chain transcripts, the translation product rapidly inactivated them, demonstrating that upon expression, the molecule was folded into a functional conformation. Ribosome inactivation by the newly-made polypeptides was indicated by the failure of ribosomes to translate a second mRNA and was supported by the observation that 28S rRNA was depurinated concomitantly. The activity of polypeptides synthesised in vitro from a synthetic ricin A chain encoding template was thus assessed in vitro at two levels; 1) Inhibition of translation, 2) Depurination of 28S rRNA. Although it has not been shown conclusively that the latter is causative of the former, it is clear (see section 6:2:3), that they are closely linked functions of ricin A chain. This system is summarised below:

Recently, it has been shown that double-stranded RNA as a contaminant of in vitro synthesised RNA is a powerful inhibitor of translation (Farrell et al., 1988). This has an obvious relevance to this present study. It is therefore necessary to equate any inhibition of protein synthesis after ricin A chain mRNA translation with 28S rRNA depurination. In this way, the inhibition observed can be attributed to the specific action of ricin A chain expression and not due to the presence of dsRNA generated in the transcription reaction.

It could be assumed that the in vitro system would function effectively with transcripts encoding any ricin - like plant or bacterial toxin which specifically modifies 28S rRNA. The major requirement would be that the translating ribosomes are sensitive to the amount of toxin generated. With sensitive ribosomes, such as those

from rabbit reticulocyte or yeast, the potency of ricin A chain is such that complete ribosome inactivation occured even when the translation product was barely detectable as a radiolabelled band on a polyacrylamide gel.

Originally, this was described as an "assay" system. However, the activity of the expressed protein was not quantified and consequently the method cannot be used as, or called an assay. Attempts were not made to quantify the amount of ricin A chain synthesised since the whole purpose of this approach was to provide a very rapid, simple assessment system without the need for lengthy purification procedures and all that would entail. Furthermore, any attempts to quantify what must amount to no more than picogram levels of protein would, in my opinion, yield data of insignificant validity at the level of resolution of techniques currently available. Purification procedures available at present would not be suitable for the efficient recovery of such ricin concentrations.

Instead, since the biological activity of, wild type ricin A chain had been demonstrated upon its expression in vitro. attention was focussed on putting this system to use in the analysis of mutant ricin A chain synthesised identically. It was envisaged that as a result of the simplicity of the system, a great many mutants could be investigated functionally in a much shorter space of time than would be possible by conventional means. It was proposed that the system be used to assess changes in the catalytic activity of ricin A chainencoded by changes in its nucleotide sequence. Chapters 7, 8 and

9 explore the potential of this system for the rapid analysis of mutant polypeptides. Mutants were generated by restriction enzyme deletion, (chapter 7), oligonucleotide - directed mutagenesis, (chapter 8), or 5' - terminal deletion of the cDNA, (chapter 9), followed by in vitro transcription. The biological activity of mutants was assessed using the system described in this chapter.

It should be stressed that it is necessary to equate any 'inhibition' of subsequent translation after A chain translation with 28S rRNA depurination. By demonstrating this, inhibition of translation would not be due to, for example, ds RNA generated in the transcription reaction as observed by Farrell and colleagues (Farrell et al., 1988).

CHAPTER 7.

AN INVESTIGATION OF THE cDNA SEQUENCE HOMOLOGY BETWEEN RICIN A CHAIN AND HAMSTER ELONGATION FACTOR 2 (EF - 2).

SECTION 7:1. INTRODUCTION.

Ricin A chain catalytically inactivates the 60S ribosomal subunit, (Olsnes and Pihl, 1972a, 1972b, Montanaro et al., 1973, Sperti et al., 1973), by a process that does not require cofactors, (Montanaro et al., 1973, Olsnes et al., 1973.). The effect of modifying the ribosome is to reduce the binding of elongation factor 2 (EF - 2) (Sperti et al., 1973), reduced GTPase activity (Benson et al., 1975). The simultaneous inactivation of these ribosomal functions can possibly be accounted for by the known interrelationship of the A ribosomal site and the EF - 2 binding site on the ribosome, (Carrasco and Vazquez, 1973, Cabrer et al., 1975). Prevention of the formation of the EF - 2 - GTP - ribosome complex by ricin A chain would thereby arrest the elongation reaction of protein synthesis. The A chain is thought to modify a site on the ribosome close to or at the binding site of EF - 2 because pre - bound EF - 2 protects ribosomes against inactivation (Fernandez - Puentes et al., 1975). Similarly EF - 2 does not bind to ricin-treated ribosomes under conditions where it did bind to intact untreated ribosomes. Further evidence which supports this theory is discussed in chapter 10, section 10:1:3.

The complete amino acid sequence of a mammalian EF - 2 has been deduced from the nucleotide sequence of a hamster EF - 2 cDNA clone (Kohno et al., 1986, Nakanishi et al., 1988.). The N - terminal

portion of this EF - 2 shows homology with GTP - binding proteins. The C - terminal half contains several regions which have homology with its bacterial counterpart, elongation factor G (EF - G), suggesting that this region of EF - 2 has been conserved during evolution and may interact with the ribosome. The possibility that ricin A chain and EF - 2 compete for the same binding site on the 60S subunit prompted us to search for primary sequence homology between the two proteins.

Some homology was found in a short stretch of DNA between bases 223 - 240 on the ricin cDNA sequence and bases 373 - 400 on the hamster EF - 2 cDNA sequence. Furthermore, this sequence showed the same homology with a corresponding region of trichosanthin. Trichosanthin is a single - chain ribosome inactivating protein derived from the root tuber of Trichosanthes kirilowii, maxim., which shows considerable sequence homology with ricin A chain (Xuejan and Jiahuai, 1986). The three protein sequences are shown below to demonstrate this homology, in figure 7.1.

Figure 7.1.

The rest of this chapter describes the experimental investigation of this homology by the deletion of this sequence from ricin A chain cDNA, (performed by P. Krieg,), the assessment of activity of the mutant polypeptide, through to an interpretation of the data obtained.

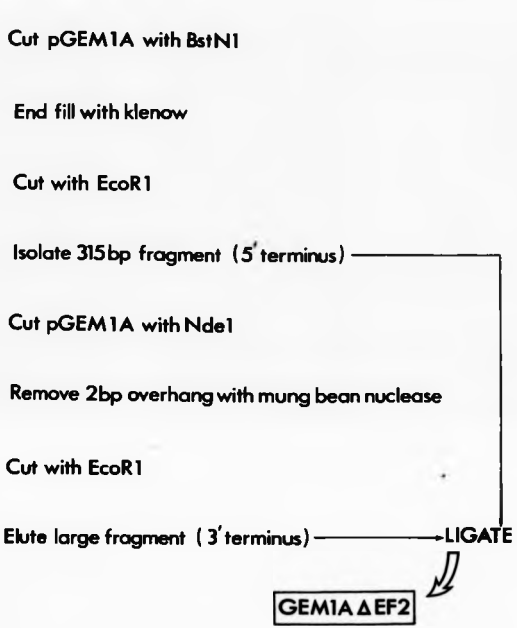
SECTION 7.2. RESULTS.

7.2.1. Deletion of base - pairs 221 - 240 of the ricin cDNA sequence using restriction enzymes.

Six amino acids showing homology with hamster EF - 2 (DVTNAY),deduced from its cDNA sequence, could be conveniently removed from the ricin cDNA by simple restriction digestion. The ricin A chain cDNA cloned in pGEM 1 was used for this work so that direct comparisons could be drawn between the activity of the wild - type and mutant polypeptides; if identical amounts of RNA are used in the translation the only variable would be the sequence deleted, since initiation environments are the same. The restriction enzyme deletion of pGEM 1A using enzymes Bst_N1 and NdE_1, was made by P. Krieg.

Figure 7.2.1. shows the scheme of events undertaken to delete the region encoding the peptide sequence; Asp - Val - Thr - Asn - Ala - Tyr, from the ricin cDNA.

Figure 7.2.1


Cut pGEM1A with BstN1
End fill with klenow
Cut with EcoR1
Isolate 315bp fragment (5' terminus)
Cut pGEM1A with Nde1
Remove 2bp overhang with mung bean nuclease
Cut with EcoR1
Elute large fragment (3' terminus)
LIGATE
GEM1A ΔEF2

7:2:2. Expression in a wheatgerm lysate.

Transcripts were prepared, as described in section 2:9, of DNA encoding pGEM 1A and pGEM 1AΔEF2, by linearising both plasmids with Hind_III and transcription as described in section 2:9.

Transcripts were translated in a wheatgerm lysate at 29° C for one hour as described in section 2:10:1., and run on a 10% SDS - polyacrylamide gel, fluorographed and autoradiographed as described in section 2:12:2 - 2:12:4.

A typical wheatgerm translation profile of pGEM 1AΔEF2 products is shown in figure 9.2.4. for comparison with other mutant ricin A chain polypeptides.

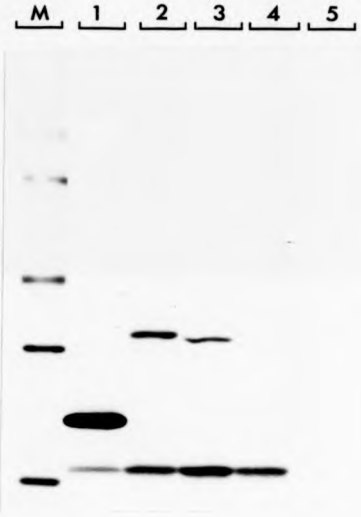
7:2:3. Expression in a rabbit reticulocyte and assessment of biological activity.

Equal amounts of the same batches of transcripts as used for the wheatgerm translation described above, were translated in a rabbit reticulocyte lysate for one hour. When 1ul of chicken lysosyme RNA was added to the reaction for a further half an hour, the samples run on a 10% SDS - polyacrylamide gel, fluorographed and autoradiographed, the results are shown in figure 7.2.2. Transcripts prepared from pGEM 1 RA EF2 translated a mutant ricin A chain that was non - inhibitory to subsequent translation, (figure 7.2.2., lane 4). Transcripts prepared from pGEM 1 RA, however, translated a protein which completely

FIGURE 7.2.2. THE ASSESSMENT OF BIOLOGICAL ACTIVITY OF PROTEIN ENCODED BY pGEM 1AΔEF2.

1µl of prepro alpha factor RNA (lane 1), ricin B chain RNA (lane 2), pGEM 1AΔEF2 RNA (lane 3), truncated A chain* RNA (lane 4) and ricin A chain RNA (lane 5), were incubated in a rabbit reticulocyte as described in section 2:11:2, for 1 hour. 1µl of chicken lysozyme RNA was then added and the reaction continued for a further 30 minutes, then 5µl samples electrophoresed on a 10% SDS - Polyacrylamide gel, fluorographed and autoradiographed as described in section 2:12. Lane M represents ^{14}C labelled molecular weight markers.

* The truncated ricin A chain (lane 4), was generated by the incorrect insertion of a stop codon into the coding sequence of ricin A chain during a site directed mutagenesis reaction. When the RNA transcribed from this cDNA was translated in a cell free system, a 10kDa protein was produced, which, by the use of this method was shown to be non toxic.


M
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5

abolished subsequent translation, (lane 5). Both ricin B chain (lane 3), and the mutant ricin A chain translation products allowed the efficient translation of chicken lysozyme RNA and both accumulated to roughly the same extent. Conversely, protein expressed from ricin A chain encoding mRNA, accumulated to a level only just detectable at the level of autoradiography and was seen to be larger than the mutant polypeptide.

Two important points concerning the toxicity of the mutant ricin A chain can be made:

1. Following translation of pGEM 1A EF2, the rabbit reticulocyte ribosomes, sensitive to wild - type A chain efficiently translated lysozyme message.

2. Ribosome inactivation did not occur, despite the greater accumulation of the mutant compared to the wild - type polypeptide.

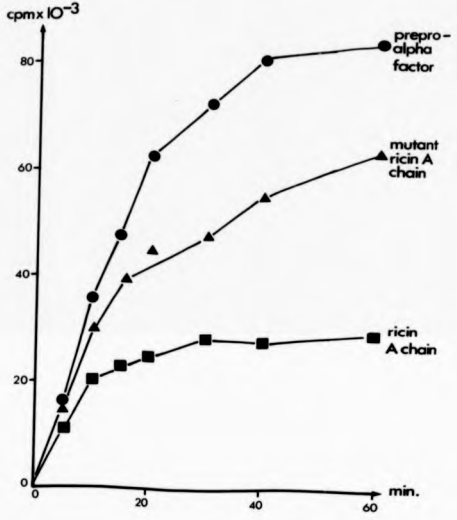
If the mutant polypeptide retained any toxic activity at all one would not expect it to show any accumulation and if accumulation were to occur, lysozyme synthesis, (ie. ribosome functioning), would certainly not be expected.

7:2:3:1. Comparison of 35S - methionine incorporation in rabbit reticulocyte lysate.

Accumulation of mutant and wild - type polypeptides were analysed further in the rabbit reticulocyte system. The rate of incorporation of 35S - methionine into TCA - precipitable protein in a translation of mutant and wild - type RNA with prepro - alpha factor as a control was followed over a time - course. Samples, (2ul), of

FIGURE 7.2.3. INCORPORATION OF ^{35}S METHIONINE INTO TCA PRECIPITABLE PROTEIN IN TRANSLATIONS OF RICIN A CHAIN MUTANT A CHAIN AND PREPROALPHA FACTOR IN A RABBIT RETICULOCYTE LYSATE.

Ricin A chain, mutant ricin A chain (with the region of homology to hamster EF2 removed), and prepro alpha factor messages (1ul), were translated for 1 hour at 37°c as described in section 2:11:2. At 5, 10, 15, 20, 30, 40 and 60 minute intervals, 2ul aliquots were removed and spotted onto Whatman 3°. 1 discs and TCA precipitated as described in section 2:11:1. Discs were counted in 4ml of Beckman EP scintillant on an LKB minibeta scintillation counter. Counts obtained were plotted against time.


cpm x 10⁻³
80
60
40
20
0
0
20
40
60
min.
prepro-alpha factor
mutant ricin A chain
ricin A chain

the translation were taken at time points as shown in figure 7.2.3. and TCA precipitated as described in section 2:10:1. Incorporation of 35S - labelled methionine into the mutant polypeptide was roughly twice that of ricin A chain and at the end of one hours translation is still increasing whereas incorporation of 35S - methionine stops after 30 minutes in the case of ricin A chain. This evidence would support the hypothesis that the ribosomes are not inactivated by the mutant polypeptide even after 1 hour in contact with twice as much protein as in the A chain translation. Ribosomes in the ricin A chain translation, on the other hand , stop translating after half an hour when the concentration of ricin A chain is only half that of the mutant polypeptide atfer one hour. In other words, ribosomes incubated in twice the amount of mutant polypeptide, for twice the length of time as wild - type ricin A chain are still active.

The conclusion drawn was that the effect of deleting six amino acids showing significant homology with those encoded by hamster EF - 2 cDNA and trichosanthin was that ribosome inactivation activity was abolished.

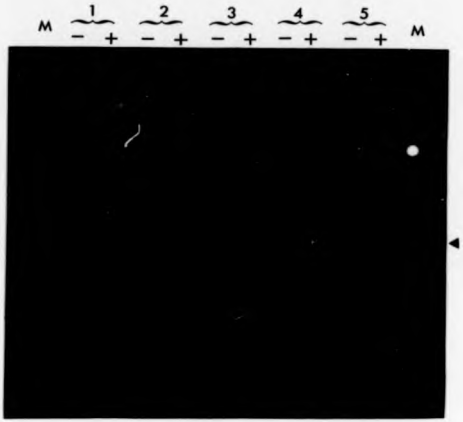
7:2:3:2. **Analysis of rRNA after translation in a reticulocyte lysate**

This work was undertaken by Dr. M.R. Hartley, to whom I am grateful.

Ribosomal RNA extracted from rabbit reticulocyte lysates which had translated pGEM 1A and pGEM 1A EF2 RNA's was analysed according to the methods described by Endo et al., (1987), and run on a formamide gel as described in section 2:4:4. Figure 7.2.4. shows ribosomal RNA's

FIGURE 7.2.4 ANALYSIS OF rRNA AFTER TRANSLATION OF MUTANT RICIN A CHAIN.

Translation mixtures were identical to those used for the experiments shown in figure 7.2.2 except that the reticulocyte lysate had not been treated with micrococcal nuclease. Following a 60 min translation of ricin A chain, mutant A chain or no RNA, ribosomal RNA was extracted, treated with aniline, as described by Endo et al., 1987, and run on a formamide gel as described in section 2:4:4. Lanes M are E. coli markers. Lane 1, no RNA added to the translation. Lane 2, ricin A chain translation. Lane 3, no added transcript, zero time control, Lane 4, translation of a transcript encoding ricin A chain from which a hexapeptide showing homology with hamster EF 2 had been deleted. Lane 5, no transcript added to the translation, but 100ng of biochemically purified ricin A chain, kindly supplied by G. Clements, added. + indicates aniline treatment, - indicates no aniline treatment. The arrow indicates the position of the 390bp fragment released by treatment of modified RNA with aniline.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M
− +
− +
− +
− +
− +

from the two translations treated subsequently with or without aniline and indicates the N - glycosidase activity of the two polypeptides. By comparison of lanes 2 and 4 it is clear that whilst pGEM 1A encodes a polypeptide with both translational inhibition activity, (figure 7.2.2. lane 5), and N - glycosidase activity,
(figure 7.2.4. lane 2), the polypeptide encoded by pGEM 1A EF2 neither inhibits translation, nor does it have a depurinating activity,
(figures 7.2.2. lane 4 and 7.2.4. lane 4, respectively.).

7.2:4. Further analysis of mutant vs. wild - type polypeptides.

The hypothesis proposed on the basis of the above evidence was that the abolition of ribosome inactivation and 28S RNA depurination was due to an inability of the mutant ricin A chain to bind to the ribosome and subsequently inactivate it. Since the region deleted showed such a strong homology with EF - 2 and trichosanthin, both of which, for their activity, must also bind to the ribosome, it may represent a ribosome binding domain.

However, one could also argue that activity was lost due simply to a loss of conformation of the tertiary structure of the protein as a result of a perturbation caused by this deletion in its primary sequence. A series of experiments was undertaken to provide supportive evidence to the hypothesis that loss of activity was due to the inability of the mutant polypeptide to inactivate ribosomes, since it could not bind to the ribosome in the first place.

7:2:4:1 Analysis of the sensitivity of mutant and wild - type ricin A chain to proteases.

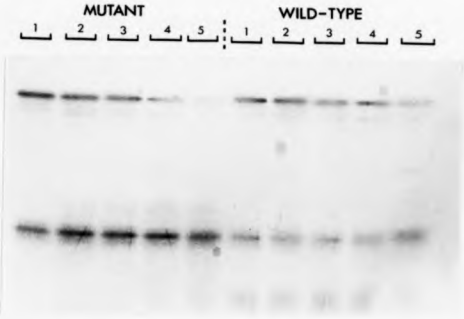
Eilers and co - workers have recently described a simple method for assessing changes in the conformation of proteins based on changes in their sensitivity to proteases during conformational changes. (Eilers *et al.*, 1988). During the import of DHFR into mitochondria its sensitivity to proteases it was normally resistant to, changed as it unfolded and then refolded. Using the same argument, if the mutant ricin A chain had undergone conformational change sufficient to cause complete loss of activity, then one could predict that such a mutant might show differential sensitivity to proteases compared to the wild - type protein synthesised under exactly the same conditions. If on the other hand, loss of activity was due to the deletion of a sequence involved in the activity of the protein, without major conformational change, then one would not predict differential sensitivity to proteases compared to wild - type A chain. The sensitivity of mutant and wild - type ricin A chain synthesised in a wheatgerm lysate to thermolysin and trypsin was compared as described in section 2:11:1. Figure 7.2.6. shows an autoradiograph of labelled polypeptides synthesised from pGEM 1A and pGEM 1AΔEF2 RNA *in vitro*. after treatment with trypsin and thermolysin followed immediately by electrophoresis on a 10% SDS - polyacrylamide gel, fluorography and autoradiography.

Figure 7.2.6.a. shows the sensitivity of mutant, (lanes 1 - 5), and wild - type, (lanes 6 - 10), ricin A chain under identical conditions to thermolysin. Figure 7.2.6.b. shows the sensitivity of

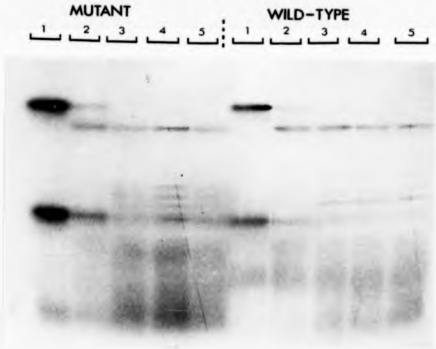
FIGURE 7.2.5 PROTEASE SUSCEPTIBILITY OF MUTANT AND WILD TYPE RICIN A CHAIN.

Ricin A chain transcripts and transcripts encoding the mutant ricin A chain were translated in a wheatgerm lysate. Samples of the translation were treated with thermolysin or trypsin as described in section 2:11:1. Lanes 1 - 5 in each case represent treatment of the sample with 0, 2.5, 5, 10 and 15ug/ml protease respectively. Figure 7.2.5a shows the susceptibility of the in vitro synthesised protein to thermolysin and 7.2.5b shows the susceptibility to trypsin upon SDS PAGE electrophoresis, fluorography and autoradiography.

a.



b.



mutant, (lanes 1 - 5), and wild - type, (lanes 6 - 10), ricin A chain under identical conditions to trypsin. Several observations can be inferred from these data;

1. Both ricin A chain and the mutant are relatively sensitive to trypsin to the same extent.

2. Both ricin A chain and the mutant are relatively insensitive to thermolysin to the same extent.

3. Cleavage products from both proteins appear at the same protease concentration,

In other words, the effect of the deletion has not been to render the protein more or less susceptible to protease attack. Thus, if this technique is a valid assessment of conformational changes of the protein, then one can say that there are no gross morphological changes in the tertiary structure of ricin A chain due to a deletion of amino acids 75 - 80. This evidence was support for the hypothesis that amino acids 75 - 80 form part of the ribosome - binding domain of ricin A chain.

Hovde and co - workers reported similar work subsequently to this, (Hovde et al., 1988). They also assumed that the change in the susceptibility of a protein to proteolytic cleavage was an indication of a change in its tertiary conformation. They showed that the substitution of aspartic acid for glutamic acid at residue 167 of the Shiga - like toxin of E.coli did not produce a major alteration in the folding of the protein since the susceptibility of the mutant to trypsin was not different to that of the wild - type. Furthermore they

trypsin was not different to that of the wild - type. Furthermore they showed that products of this digestion were identical to that described previously, (Olsnes, 1981).

SECTION 7:3. DISCUSSION.

The effect of deleting amino acids 75 - 80 from ricin A chain was to completely abolish its activity as an inhibitor of protein synthesis and as an N - glycosidase. Amino acids 75 - 80 show considerable homology with amino acids 124 - 133 of hamster EF - 2 as deduced from the cDNA sequence. Homology in this region is also shared with the ribosome inactivating protein trichosanthin. Both ricin A chain and trichosanthin catalytically inactivate eukaryotic ribosomes whilst EF - 2 also interacts with eukaryotic ribosomes during the elongation reaction of protein synthesis. All three proteins compete for the same substrate, (see section 7:1.), all show significant homology in a specific region and deletion of this region from one of the proteins, (ricin A chain), abolishes its activity without loss of tertiary structure. Circumstantial as it may be, this was evidence to suggest that the region encoded by amino acids 75 - 80 of ricin A chain is part, at least, of a ribosome - binding domain. One is assuming here that loss of activity was due to the inability of the mutant ricin A chain to bind to the ribosome. Evidence was presented to suggest that this was in fact what had happened and not

simply an inactivation due to loss of tertiary structure. Protease susceptibility experiments indicated that the deletion had not caused a major alteration in the conformation of the protein. The implications of these data for the delineation of functional domains of ricin A chain are discussed in chapter 10.

CHAPTER 8.

THE GENERATION OF RICIN A CHAIN MUTANTS BY OLIGONUCLEOTIDE - DIRECTED MUTAGENESIS AND ASSESSMENT OF THEIR BIOLOGICAL ACTIVITY in vitro.

SECTION 8:1. INTRODUCTION

Reversible chemical modification of ricin A chain has indicated that arginine residues play an important role in ribosome inactivation (Watanabe and Funatsu, 1986). There are twenty arginine residues in ricin A chain, but recent work has shown that the essential arginine residues lie at the N - terminal region which includes six argininee. A comparison of the N - terminal sequences of ricin A chain and trichosanthin, (Xuejun and Jiahuai, 1986), revealed that three of these were conserved in both proteins. The conserved residues were at positions 29, 48 and 56 in ricin A chain. Of these it was arginine 48 and 56 that appeared to lie in the three dimensional cleft proposed as the active site. (Montfort et al., 1987). See section 1:5 and figure 1.5.1.

Argininee 48 and 56, conserved between trichosanthin and ricin A chain and located in the " active cleft ", provided ideal candidates for mutagenesis to determine their role in the mechanism of action of ricin A chain.

A comparison of the sequences encoding ricin A chain and trichosanthin also revealed three conserved pentapeptides, namely SHAAR, GYRAG and LREMI, see section 1:5. The significance of the SHAAR pentapeptide is that the glutamic acid, E, is known to lie at a position in the active site cleft (J.D. Robertus, personal communication). This pentapeptide would thus also be a good choice for mutagenesis. Similarly, glutamic acid 167 of Shiga - like toxin, with which the

glutamic acid in the ricin A chain pentapeptide, SEAAR, shows positional homology, was mutated and was shown to form part of the active site of this toxin. (Hovde et al., 1988).

SECTION 8:2. RESULTS

8:2:1. **Design of a vector for in vitro expression of wild type and mutant ricin A chain sequences.**

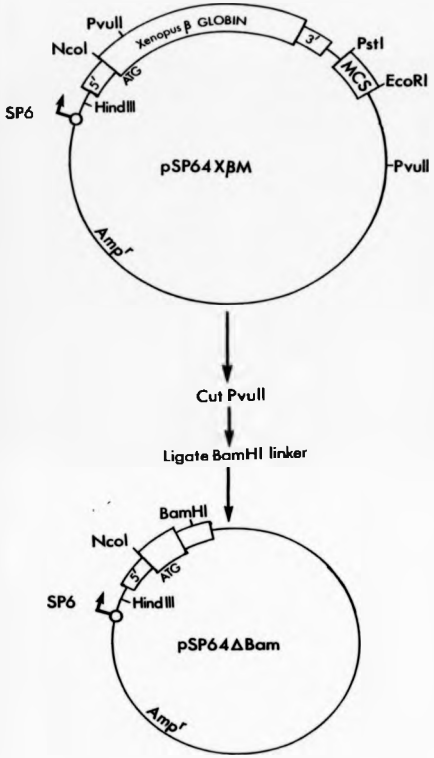
Standard oligonucleotide - directed mutagenesis of single stranded DNA requires two sets of subcloning. Firstly, from the plasmid harbouring the cDNA into the M13 vector (Messing et al., 1977) for mutagenesis, followed secondly by subcloning from an RF M13 DNA into the plasmid for expression or other analysis of the modified DNA. The restriction sites present at the 5' and 3' termini of the ricin A chain cDNA would not permit such subcloning with sufficient ease to make this approach practicable. In order to ease the problem of subcloning, a new vector was designed for the ultimate expression, transfer and recovery of mutant ricin A chain sequences.

The BamH 1 sites present at bases -42 and 852 provide suitable unique positions at which to lift out a " ricin A chain cassette " containing a ricin A chain sequence encoding a 3' stop codon but not a 5' ATG codon. For the ultimate expression of such sequences, a plasmid containing an ATG codon downstream of which the ricin A chain cassette can be cloned was needed. The vector pSP64 Xβm after suitable manipulation was considered an excellent choice for this purpose. Consultation of the Xenopus β globin cDNA sequence, (Williams et al.,

1980), revealed two Pvu II restriction sites, one 8 codons into the coding sequence and the other 85 codons into the coding sequence. Plasmid pSP64 has a single Pvu II site at position 1668. Cleavage of pSP64 XβB with Pvu II thus generated a linearised plasmid containing an SP6 promoter, (for the in vitro transcription of cloned DNA), the 5' untranslated region of the Xenopus β globin gene, (for the stability of in vitro transcripts), an ATG codon of a highly expressed gene (in a favourable initiation environment for the efficient cell - free expression of the sequence cloned in frame and downstream of it). Eight codons of the β globin gene were followed by blunt ends for cloning in the desired cDNA sequence with the ampicillin resistance gene for the selection of recombinant plasmids and a plasmid origin of replication. To enable the simple cloning of a BamH I fragment encoding ricin A chain, BamH I 12mer linkers, (5'CCCGGATCCGGG3') were ligated into the blunt ended vector to generate a new in vitro expression vector, pSP64 Δ Bam. (see figure 8.2.1.). Such a construction allows the insertion of ricin A chain cDNA cleaved from any vector using BamH I in frame with the globin ATG for in vitro transcription and assessment of activity using the system described in chapter 7. Furthermore by choosing BamH I as the linker sequence, ricin A chain sequences can be simply lifted out of this vector into the BamH I cloning site of pDS5/3 (see O' Hare et al., 1987), for subsequent E.coli expression. This approach allows the initial assessment of the activity of mutant ricin A chain in vitro, followed by large scale expression to assess quantitatively, the effect of the mutation on the activity of ricin A chain using simple manipulations to transfer the sequence of interest from on vector to another. Furthermore by rapid assessment of activity of the mutant in vitro, one can determine whether or not it would be worthwhile to commit time and

FIGURE 8.2.1 CONSTRUCTION OF pSP64ΔBam

The bulk of Xenopus β globin coding sequence, the 3' untranslated region and MCS was removed by digesting pSP64Xβm with Pvu II. A Bam HI linker (CCCGGATCCGGG) was ligated to recircularise the vector pSP64ΔBam.


PvuII
NcoI
Xenopus β GLOBIN
3'
PstI
5'
ATG
MCS
EcoRI
SP6
HindIII
pSP64XβM
PvuII
Ampr
Cut PvuII
Ligate BamHI linker
BamHI
NcoI
5'
ATG
SP6
HindIII
pSP64ΔBam
Ampr

resources to subsequent E. coli expression. Other advantages are obvious:

1. The rapid and efficient translation of ricin A chain template from an ATG in a suitable initiation environment allowing maximal expression in the minimum time to suit the requirements laid down in chapter 6.

2. If both wild type and mutant cDNA sequences are transcribed from the same vector, valid direct comparisons can be made between the data obtained as each is assessed separately. By using such a system, a degree of uniformity is obtained. Wild type ricin A chain for this purpose was cut from pDS5/3 A (O' Hare et al., 1987), using BamH I and also from pGEM 1A using BamH I. The 5' terminal sequence of these two clones is shown in figure 8.2.2.

A restriction digest to show the correct orientation of ricin A chain cDNA cloned in pSP64ΔBam is shown in figure 8.2.3b

8.2.2. Site - directed mutagenesis.

A ricin A chain sequence was cut from plasmid pGEM 1A using Hind III and EcoR I and inserted into M13 mp18 cut with the same enzymes. The orientation (shown in figure 8.2.3a) of the A chain sequence in this vector was such that antisense oligonucleotide primers were used for mutagenesis. These were kindly provided by ICI, Alderley Edge, Cheshire. The following sequences were proposed for mutagenesis:

1. DNA 5' TTG CCA AAC AGA GTT GGT TTG CCT ATA 3'

   OLIGO 3' AC GGT TTG CGC CAA CCA AA 5'

   19mer, 3 base mismatch, converts Arg 48 to Ala 48

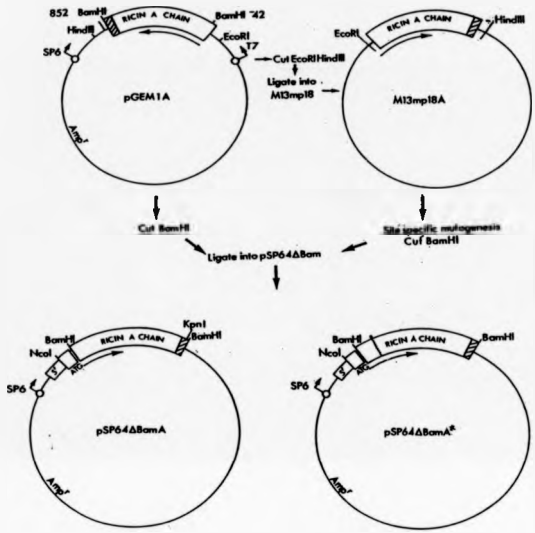


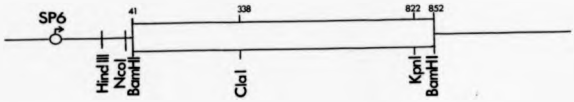
FIGURE 8.2.3a CLONING RICIN A CHAIN IN pSP64 Bam

For *in vitro* transcription the ricin A chain sequence was excised from pGEM 1A as a *Bam HI* fragment and ligated into pSP64 Bam. For oligonucleotide site directed mutagenesis, a *Hind III Eco RI* fragment was isolated and ligated into M13mp18. After mutagenesis, the altered A chain fragments were excised as *Bam HI* fragments and ligated into pSP64 Bam. The hatched region represents the stretch of ricin sequence downstream of the inserted A chain stop codon

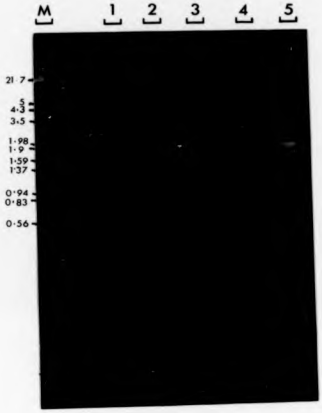
FIGURE 8.2.1 THE 5' TERMINAL SEQUENCE OF THE FUSION PROTEIN CREATED BY LIGATING RICIN A CHAIN cDNA IN pSP64ΔBam.

FIGURE 8.2.3b RESTRICTION ENDONUCLEASE ANALYSIS OF pSP64ΔBam A

Plasmid pSP64ΔBam A, lane 5, was digested with; Nco I Cla I. lane 1; Nco I Kpn I. lane 2; Hind III Cla I. lane 3; Bam HI. lane 4, lane M represents a Hind III EcoR I digest of lambda DNA. The reactions were electrophoresed on a 1% agarose gel. A restriction map of pSP64Δ Bam A is shown below for reference.



pSP64ΔBam = 2·9kb


M
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2
3
4
5
21·7
5
4·3
3·5
1·98
1·9
1·59
1·37
0·94
0·83
0·56

2. DNA 5' TTG CCT ATA AAC CAA CGG TTT ATT TTA GTT G 3'
   OLIGO 3' C GGA TAT TTG GTT CGT AAA TAA AAT CAA C 5'
   29mer, 3 base mismatch, converts Arg 56 to Ala 56

3. DNA 5' TTG CCT ATA AAC CAA CGG TTT ATT TTA GTT G 3'
   OLIGO 3' C GGA TAT TTG GTT --- AAA TAA AAT CAA C 5'
   26mer, loops out Arg 56

4. DNA 5' CTT GCT GGT AAT CTG AGA GAA AAT ATC GAG TTG GGA AAT 3'
   OLIGO 3' GAA CGA CCA TTA --- --- --- --- --- CTC AAC CCA TTA 5'
   24mer, loops out LRENI

5. DNA 5' ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT 3'
   OLIGO 3' TAG GTT TAC TAA --- --- --- --- --- AAG GTT ATA TAA 5'
   24mer, loops out SEAAR

6. DNA 5' AGA GCT GTT CGC GGT CGT TTA ACA ACT GGA GCT 3'
   OLIGO 3' CT CGA CAA --- CCA --- AAT TGT TGA CCT CGA 5'
   25mer, loops out Arg 29 and 31

The sequences, LRENI and SEAAR, refer to two pentameric amino acid sequences identified by Xuejun and Jiahuai (1986), as showing significant homology between ricin A chain and the ribosome inactivating protein, trichosanthin.

Mutagenic oligonucleotides were labelled with ^{32}P ATP by kinasing as described in section 2:8:3. It was necessary to purify the oligonucleotides for the conversion of Arginines 56 and 48. This was achieved by their elution from a 16% Poly acrylamide sequencing gel as described in section 2:6:2. The two oligonucleotides were re - kinased as above and are shown in figure 8.2.4., labelled oligonucleotides 3 to 6, above are shown in figure 8.2.5.

FIGURE 8.2.4 PURIFICATION OF MUTAGENIC OLIGONUCLEOTIDES

Two of the oligonucleotides supplied for mutagenesis were supplied contaminated with a series of smaller oligonucleotides, lanes 2 and 4. Purification of the full length oligonucleotide was carried out as described in section 2:6:2. After rekinasing the oligonucleotides as described in section 2:8:7, they were run on a 16% sequencing gel as described in section 2:4:5 and visualised by autoradiography, lanes 1 and 4. Lanes 1 and 2 show pure and impure oligo Arg 56 whilst lanes 3 and 4 show impure and pure oligo Arg 48, respectively.

Arg 48 5' TTG CCA AAC AGA GTT GGT TTG CCT 3' DNA
(Arg)

Ala 48 3' AC GGT TTG CGC CAACCA AA 5' OLIGO
(48, Ala)

Arg 56 5' GGT TTG CCT ATA AAC CAA CGG TTT ATT TTA GTT G 3' DNA
(Arg)

Ala 56 3' C GGATAT TTG GTT GCT AAATAAAATCAA C 5' OLIGO
(56, Ala)

FIGURE 8.2.5 KINASED MUTAGENIC OLIGONUCLEOTIDES

Mutagenic oligonucleotides were kinased using ^{32}P ATP as described in section 2:8:7 and run on a 16% sequencing gel as described in section 2:4:5. Lane 1 = loop out Arg 56

Lane 2 = loop out SEAAR

Lane 3 = loop out LREMI

Lane 4 = loop out GYRAG


1
2
3
4

8:2:2:1 Screening positive clones.

Site - directed mutagenesis was carried out exactly as described in section 2:8, using the oligonucleotides described above. Screening the clones obtained was carried out as described in section 2:8:8, by washing ^{32}P labelled oligonucleotides hybridised to plaques immobilised onto nitrocellulose filters at 5°c below the dissociation temperature (T_{d-5}), and at the dissociation temperature (T_d) calculated according to the Wallace rules, (Suggs et al., 1981), for each oligonucleotide. The autoradiographs obtained for T_d washes of oligonucleotides 1, 2, 3 and 5 are shown in figures 8.2.6 and 8.2.7.

8:2:2:2. Sequencing positive clones.

Colonies to which the respective radiolabelled oligonucleotide hybridised strongly at the T_d as shown in figures 8.2.6 and 8.2.7 were considered to represent positive mutants, (positives). The exact identity of the mutant sequence was confirmed by sequencing single stranded DNA prepared (as described in section 2:3:3), from plaque purified positives (see section 2:8:8), using ^{35}S - ATP and run on an 8% buffer gradient gel (Biggin et al., 1983), as described in section 2:4:5:2. From sequence data obtained, the successful conversion of Arg 48 to Ala 48, Arg 56 to Ala 56, the removal of Arg 56 and the removal of SRAAR was confirmed. Sequences are shown in figures 8.2.8 to 8.2.11, against the wild type sequence and the sequence described by Lamb et al., (1985), for reference. Putative positives were obtained for the removal of Arg 29 and 31 and the removal of the pentamer LRSNI, however, although a number (8 in each case) of putative positives were sequenced in each case none were found to represent mutant sequences. It was not considered worthwhile to commit any further effort to obtaining these mutants, instead, confirmed mutant

FIGURES 8.2.6 and 8.2.7 SCREENING MUTANT COLONIES

Site directed mutagenesis was carried out as described in section 2:8, using the oligonucleotides shown in figs 8.2.4 and 8.2.5. Screening was carried out as described in section 2:8:8. The figures show the Id washes for each of the mutants which were subsequently analysed.

a. Arg 56 ➡ Ala 56

b. Arg 48 ➡ Ala 48

Arg56 loop out

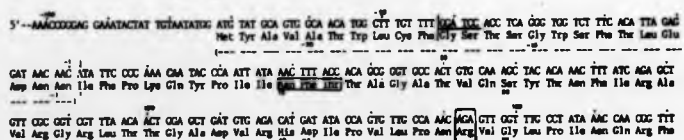
a.

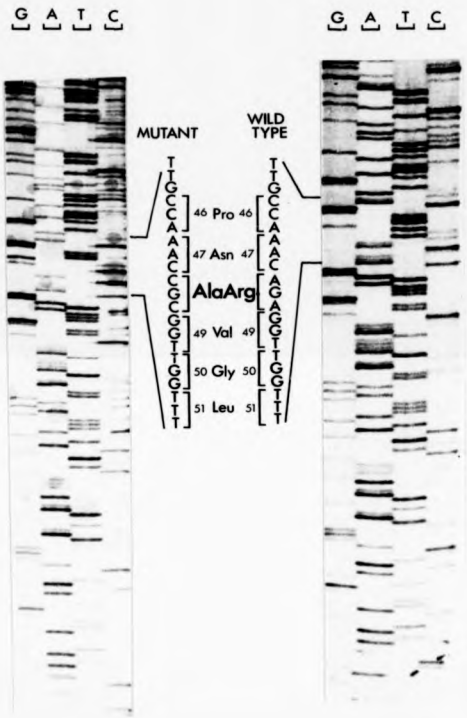
SEAAR loop out

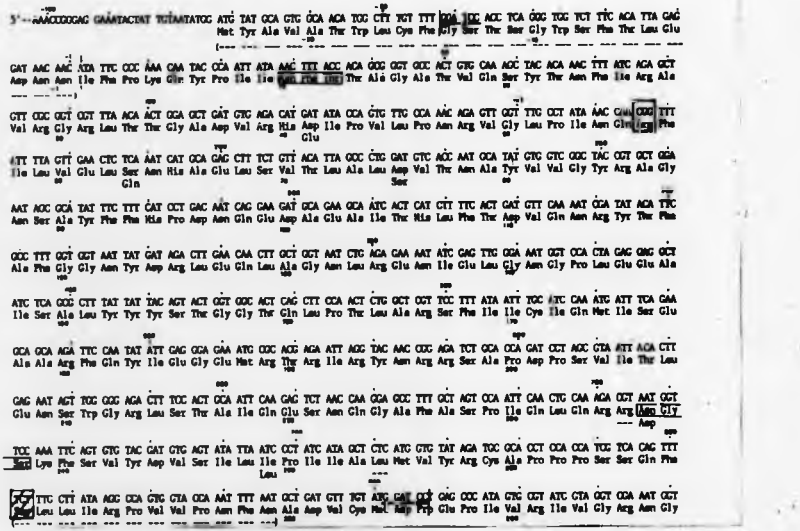
b.

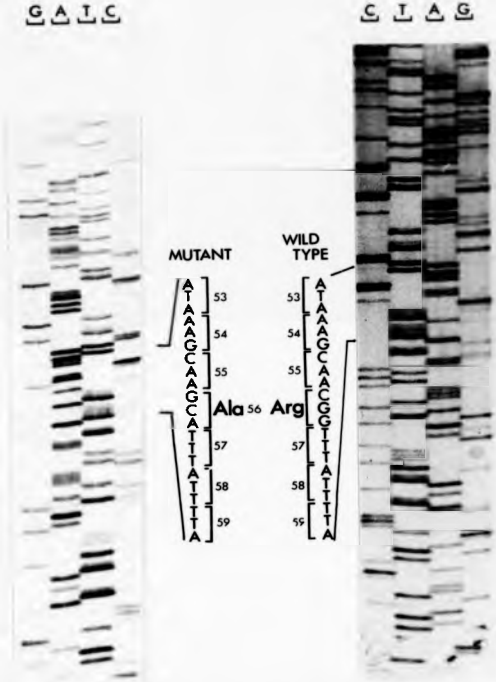
FIGURES 8.2.6 - 8.2.11 SEQUENCING MUTANTS

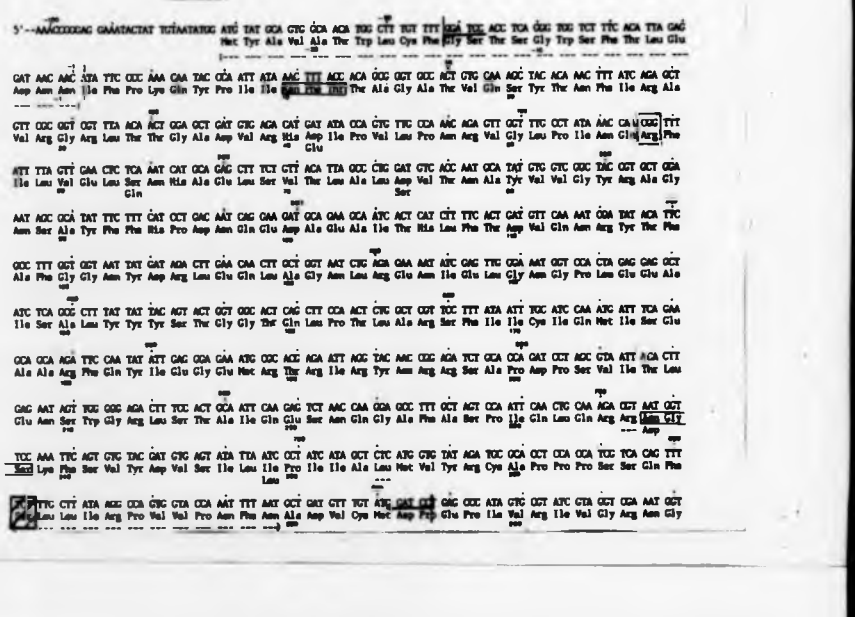
The positive clones shown in figures 8.2.6 and 8.2.7 were plaque purified and sequenced as described in section 2:8:2. In each figure the tracing paper overlay shows the 5' and 3' Bam HI sites used to excise the ricin A chain coding sequence, the 3' terminal stop codon (hatched box) and the sequence modified (open box). Dashed underlining on the overlay represents the 5' signal sequence and 3' linker region of the ricin A chain sequence. Boxed amino acids represent putative N-glycosylation sequences proposed by Lamb et al., 1985.

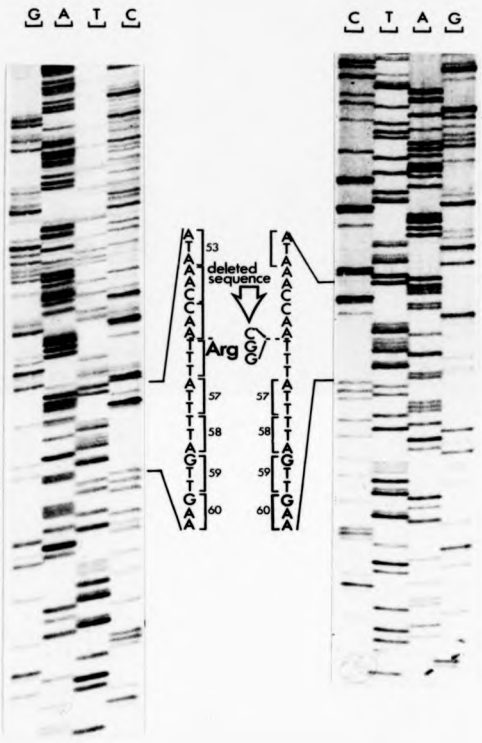


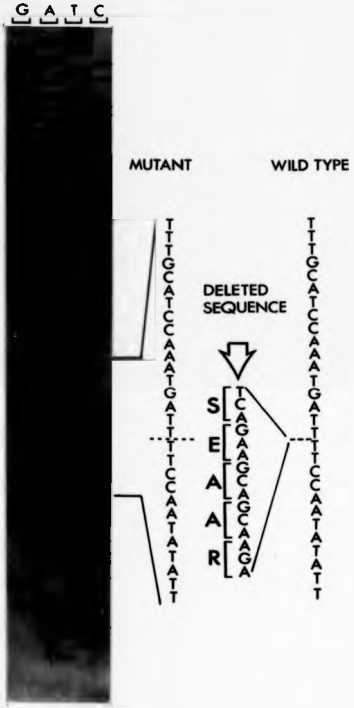

G A T C
G A T C
MUTANT
WILD TYPE
T T G C C A A A C C G C G G T T G G T T T
T T G C C A A A C A G A G G T T G G T T T
46 Pro 46
47 Asn 47
AlaArg
49 Val 49
50 Gly 50
51 Leu 51




G A T C
C T A G
MUTANT
WILD TYPE
ATAAAGCAAGCATTTATTTTA
ATAAAGCAACGGTTTATTTTA
53
54
55
Ala 56
Arg
57
58
59




G A T C
C T A G
53
deleted sequence
Arg
57
58
59
60


G A T C
MUTANT
WILD TYPE
TTTGCATCCAAATGATTTTCCAATATATT
DELETED SEQUENCE
S E A A R
TCAGAAGCAGCAAGA
TTTGCATCCAAATGATTTTCCAATATATT

sequences portrayed in figures 8.2.8 to 8.2.11, were subcloned into pSP64 Bam for the assessment of their activity.

8:2:2:3. Subcloning confirmed mutant sequences into pSP64 Bam.

RF DNA preps of the four mutants were made as described in section 2:3:4 and cut with Bam_HI. Mutant ricin A chain encoding 810 bp fragments were gel eluted (see section 2:6:1:2), phenol extracted and ethanol precipitated, then cloned into Bam_HI cut pSP64 Bam. The correct orientation of inserts into the vector was confirmed by restriction digestion, see figure 8.2.3. For transcription, large preps of plasmids with inserts in the correct orientation were made as described in section 2:3:1, and linearised with Kpn I, which cuts in the linker region of the ricin cDNA at position 822. Transcription was carried out exactly as described in section 2:10, using ^3H - labelled UTP, to allow quantification of the transcription reaction. Successful transcription was not assessed by running labelled transcripts on formaldehyde gels, but instead was evaluated by translation in a wheatgerm lysate.

8:2:3 Wheatgerm lysate translation of mutant ricin A chain RNA

Wheatgerm translations were carried out exactly as described in section 2:11:1. For comparison with the wheatgerm translation products of other mutant ricin A chain templates, translation products were run on a 10% SDS - polyacrylamide gel, fluorographed and autoradiographed as shown in figure 9.2.4. Compared to wild type ricin A chain (lane 1), Ala 48, Ala 56 and 56, were all of identical size as predicted. (lanes 3 - 5), whilst the SEAAR

deletion, (lane 6), was a similar size to the products of pGEM1 A EF2, (lane 2), which lack 5 and 6 amino acids respectively. These data indicated that the mutant ricin A chains were efficiently translated and of the predicted size. Furthermore, they also indicated importantly that the Xenopus ATG codon, when fused to the 5' terminus of ricin A chain cDNA efficiently directed the translation of the A chain in vitro and that all of the sequences translated from this ATG were expressed to roughly the same extent.

Efficient expression in a wheatgerm system confirmed that translation products were of the predicted size before proceeding with the assessment of their toxicity in a rabbit reticulocyte lysate where their visible accumulation would not be predicted if still catalytically active.

8:2:4 Assessment of the biological activity of mutant and wild type ricin A chain polypeptides.

RNA template encoding each of the four mutants, ricin A chain, prepro - alpha factor and ricin B chain were translated in a rabbit reticulocyte lysate for one hour. Quantification of the amount of RNA made was carried out as above and equal quantities of template were added to each translation to allow comparisons to be drawn between each of the four mutants and wild type ricin A chain upon expression. An aliquot of 1ul of chicken lysosyme RNA was added to each translation and incubation continued for a further 30 min. At the end of this time, 5ul aliquots were loaded onto a 10% SDS Polyacrylamide gel and electrophoresed for 16 hours, fluorographed and

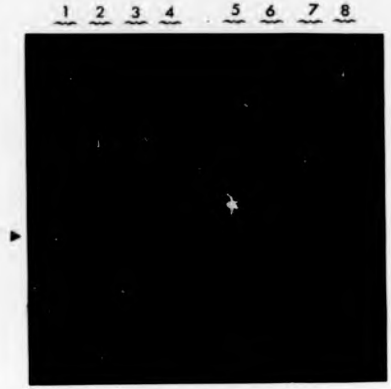
autoradiographed as described in section 2:12:3 . This autoradiograph is shown in figure 9.2.6.

8.2.5 Analysis of rabbit reticulocyte 28s RNA after translation of mutant ricin A chain sequences.

This work was undertaken by Martin Hartley, to whom I am grateful. Ribosomal RNA extracted from rabbit reticulocyte lysates which had translated mRNA transcribed from pSP64ΔBam A and mutants Ala 48 and Ala 56, cloned in pSP64ΔBam, was analysed according to the methods described by Endo and colleagues (Endo et al., 1987). Figure 8.2.12 shows ribosomal RNA's from the three translations treated subsequently with or without aniline and run on a formamide gel as described in section 2:4:4 and indicates the N - glycosidase activity of the wild type and mutant polypeptides. It is clear that both of the mutants analysed show N - glycosidase activity as well as the ability to inhibit translation. This would also indicate that the observed inhibition of protein synthesis is a demonstration of the biological activity of the expressed protein and not due to that caused by dsRNA contamination in the template sample. Such an analysis was also carried out for the other mutants described with the same result. (data not shown).

FIGURE 8.2.12 ANALYSIS OF 28S rRNA AFTER THE TRANSLATION OF MUTANT RICIN A CHAIN IN A RABBIT RETICULOCYTE LYSATE

The effect of converting arginine residues at positions 48 and 56 to alanine on the rRNA modifying activity of ricin A chain was demonstrated in exactly the same way as in fig 7.2.4. Lane Plastocyanin ferredoxin reductase transcripts as a control, supplied by C. Robinson. Lane 2, Arg 48 - Ala; Lane 3, Arg 56 - Ala; Lane 4, ricin A chain transcripts. Samples loaded in lanes 1 - 4 were treated with aniline, whereas exactly the same samples, without aniline treatment were loaded in lanes 5 - 8. The arrow indicates the 390 nucleotide fragment released by aniline treatment of modified RNA.


1 2 3 4 5 6 7 8

SECTION 8.3. SUMMARY.

Several conclusions can be drawn from these data

1. Expression of wild type ricin A chain sequences

Ricin A chain expressed from pSP64 Bam was functional despite it being a fusion protein with 5 amino acids from the vector pDS5/3 (see O' Hare et al., 1987), 8 amino acids from the Xenopus B globin gene and four from the C- terminus of the ricin signal peptide and a proline donated by the Bam HI linker. The N - terminus of this fusion protein is shown in figure 8.2.1.

2. The conversion of Arginines 48 and 56 to Alanines, the complete removal of Arginine 56 and a region encoding the amino acid sequence SEAAR were all without effect upon the inhibition of protein synthesis by rabbit reticulocyte ribosomes or on the de purinating activity of the protein. In other words, all of the mutants remained catalytically active, despite, in the case of SEAAR, the removal of 5 amino acids from the protein. Furthermore, despite the implications of the work of Funatsu, and despite the strong homologies with trichosanthin, (Xuejan and Jiahuai 1987), abrin, (Funatsu, et al., 1988) and Shiga-like toxin (Endo et al., 1988), these regions do not appear to have any major role in the catalytic activity of ricin A chain. This was surprising, particularly in the light of the work of Hovde et al., (1988) in which, by similar mutagenesis of the Shiga - like toxin gene, glutamic acid 167, homologous with the glutamic acid in SEAAR, was shown to play a major role in the catalytic activity of that toxin which shares the same mechanism of action as ricin A chain.

3. A further point to make is that the activity of the expressed mutant polypeptide can be assessed only 6 days after identifying the

mutation by sequencing, emphasising the value of this approach over conventional methods for the evaluation of mutagenesis on the activity of the gene of interest.

Site directed mutagenesis of ricin A chain cDNA yielded four mutants which were identified by sequencing. Transcripts synthesised in vitro from the SP6 promoter of a specially designed vector pSP64 ΔBam, were translated in a wheatgerm lysate and generated proteins of the predicted size. When these polypeptides were expressed in a rabbit reticulocyte lysate, the mutations in the cDNA were found to have no effect upon the catalytic or depurinating activity of the proteins; they were as active as the wild type.

Further discussion of these data will be reserved till chapter 10 for their evaluation in the light of the results of chapter 9.

CHAPTER 9.

THE GENERATION OF N - TERMINAL DELETION MUTANTS OF RICIN A CHAIN BY EXONUCLEASE III DIGESTION OF THE cDNA AND ASSESSMENT OF THEIR BIOLOGICAL ACTIVITY in vitro.

SECTION 9:1. INTRODUCTION.

Ricin A chain is one of the ribosome inactivating toxins upon which attention has been focused for the construction of immunotoxins (Vitetta *et al.*, 1987). One major problem which arises when such molecules are used in *in vivo* cancer therapy, is the development of a host antibody response against both the toxin and the monoclonal antibody (see section 1:6) . The immunogenicity of the antibody may be reduced by using human monoclonals or by humanising murine antibodies. In the case of the toxin moiety, one approach to this problem would be to define ricin A chain fragments of minimum size which still retain full catalytic activity. To this end, a series of deletion experiments was proposed. Not only can such an approach define the minimum genetic structure required for recombinant toxin to be active, it can also, at the same time identify the functional domains of the molecule. The value of this technique in both the above respects is evident from studies made on *Pseudomonas* exotoxin A, (PA), (Hwang *et al.*, 1987). *Pseudomonas* exotoxin A makes an extremely active immunotoxin when conjugated to an antibody (Fitzgerald *et al.*, 1983a, 1983b). However problems arise during *in vivo* treatment using such conjugates as a result of the chemical derivatisation procedures used in their production. In order to achieve the highest degree of killing of tumour cells it is clearly desirable to administer as large amounts of immunotoxins as possible. A recombinant DNA approach to immunotoxin production was used in this case to overcome the reliance on chemical inactivation. Furthermore, high level expression of cloned *Pseudomonas* exotoxin A provides ample material for maximised tumour cell killing. By deleting portions of

the cloned gene and subsequent expression, (Gray *et al.*, 1984) and assay, Hwang and co - workers were able to identify the function of each of the three domains of the PE gene determined previously by X - Ray crystallography (Allured *et al.*, 1986). The cell recognition domain was deleted and the modified Pseudomonas toxin gene was fused to the transforming growth factor type α gene. The chimaeric protein was expressed in *E. coli* and purified. It was suggested that this chimaeric protein might be useful in treating cancers that contain high numbers of EGF receptors. (Chaudhary *et al.*, 1987).

This approach is typical of the current research in the field of immunotoxins.

1. Chemical coupling of toxin and antibody.
2. Identification of problems arising through this approach.
3. Recombinant DNA approach.
   a) Cloning the gene.
   b) Identification of functional domains of the toxin gene.
   c) Deletion of gene to produce the minimum genetic material to encode full toxin activity.
   d) *In vivo* assay.

With the reported success of the deletion approach for producing reduced - size immunotoxins, a similar strategy was planned for ricin A chain using the enzyme Exonuclease III to produce a series of 5' - terminal deletions (Rogers and Weiss, 1980, Henikoff, 1984.).

By using the toxicity assessment system described in chapter 7 it would be possible to test a great many mutants in a relatively short space of time.

SECTION 9:2 RESULTS

9:2:1 **Design of a vector for the in vitro transcription and translation of wild type and mutant ricin A chain sequences.**

Following 5' terminal deletion of the ricin A chain cDNA, the 5' terminus would be blunt and without an ATG codon. It would be quite feasible to clone a wholely blunt-ended ricin A chain fragment into the vector pSP64 XβB cleaved with Pvu II. However, this approach would impose severe constraints upon the recovery of deletions with a coding sequence in-frame with the Xenopus β globin ATG codon. Furthermore, if a blunt-ended set of fragments were ligated into Pvu II cut pSP64 XβB, not only would only one - third of the deleted sequences be in-frame with the ATG codon, but also the fragments could be ligated in to the vector in two orientations, reducing the probability of obtaining an in-frame deleted sequence in the correct orientation to about 15%. Thus, in order to increase the probability of obtaining sequences in-frame with the ATG codon, to at best, 33%, it was considered necessary to force the ligation of the 3' terminus of the inserts by making use of the restriction enzyme sites downstream of the Xenopus globin gene in pSP64 XBm. The following scheme of events was proposed:

A. **Preparation of vector.**

1. Cut pSP64 XBm with Hae I.
2. End fill, to generate the sequence C C A T G
3. Cut with Pst I

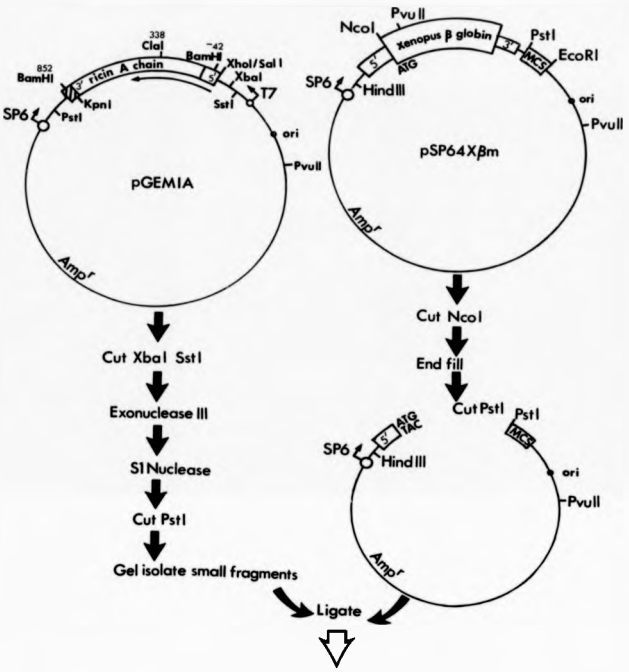


FIGURE 9.2.1

1. Ricin A chain sequences in frame with Xenopus ATG identified by translation of RNA template in wheatgerm.

2. Confirm identity of deleted sequences by plasmid sequencing.

3. Assess catalytic activity of mutants in rabbit reticulocyte lysate

.....

B. Preparation of insert: Exonuclease III deletion.

1. Cut pGEM 1A with Xba I and Sst I.
2. Incubate with Exonuclease III (see section 2:9)
3. Remove overhangs with S1 nuclease.
4. Cut with Pst I.
5. Gel isolate small fragments.
6. Ligate into vector prepared as above.

This scheme of events was carried out exactly as described in section 2:9 and is summarised in figure 9.2.1. Products of the deletion reaction, after S1 nuclease treatment were run on a 1.5% low melting point agarose gel which is shown in figure 9.2.2.

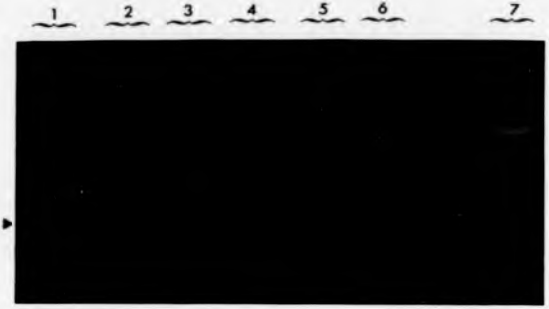
9:2:2. Cloning the deleted sequences.

Five prime deleted sequences encoding ricin A chain with a Pst I overhang on the 3' end were eluted from a 1% low melting point agarose gel as described in section 2:6:1:2 . Samples were purified by phenol extraction and concentrated by ethanol precipitation and then ligated into Nco I cut, end filled, Pst I cut pSP64 Xβm. This ligation reaction was used to transform E. coli cells, as described in section 2:7:2, from this, 24 small - scale plasmid preps were prepared as described in section 2:3:2 . To check that inserts had been ligated in the correct orientation for the subsequent synthesis of sense transcripts from the SP6 promoter, one of the plasmids obtained was digested with restriction enzymes and run on a 1% agarose gel as shown in figure 9.2.3. By filling in the Nco I site and ligating in a blunt ended fragment it was seen (lane 2), that the Nco I site was

FIGURE 9.2.2 LOW MELTING POINT AGAROSE GEL ELECTROPHORESIS OF DELETED RICIN A CHAIN cDNA

Products of the exonuclease III deletion reaction were removed at 0 secs lane 6; 30, lane 5; 60, lane 4; 90, lane 3; 120, lane 2; and 150 secs, lane 1. The reaction mix was phenol extracted, blunt ended with S1 nuclease and cleaved with Pst I. The whole reaction was loaded on a 1.5% low melting point agarose gel and electrophoresed. The deleted fragments (indicated by the arrow), were subsequently ligated into the vector shown in figure 9.2.1. Lane 7 is Cla I Pst I cut pGEM IA the lower band is roughly 550bp and its size acted as a indication of the extent of the deletion reaction.

See for reference figure 3.2.1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6
7

FIGURE 9.2.3 RESTRICTION ENDONUCLEASE ANALYSIS OF DELETION MUTANTS

For the purposes of demonstrating that the deleted ricin A chain fragment had been inserted in the vector in the correct orientation, they were cut with restriction enzymes. One of them is shown here. Lane 1 = deleted A chain in transcription vector

Lane 2 = Nco I digest

Lane 3 = Hind III Bam HI digest

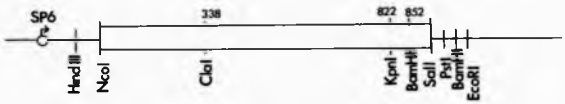
Lane 4 = Hind III Bam HI digest

Lane 5 = Bam HI digest

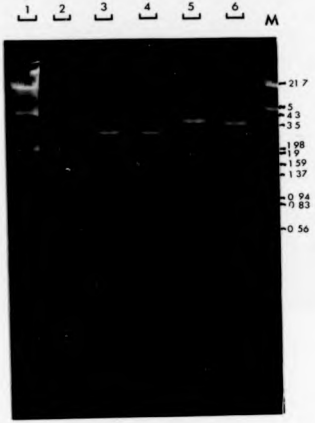
Lane 6 = Hind III Cla I digest

M = Hind III Eco RI digest of lambda DNA

A restriction map of the 5' deleted fragments cloned in the in vitro expression vector is shown below for reference.



Vector = 3kb


1
2
3
4
5
6
M
21 7
43
35
198
159
137
0 94
0 83
0 56

destroyed as would be expected. Lane 5, representing a Bam HI digest showed that of the two Bam HI sites in the A chain sequence, 1 had been destroyed leaving only the 3' terminal site and the site in the polylinker of the vector, the roughly 50bp fragment generated by this digest was not visible on a 1% agarose gel. The correct orientation of the insert was confirmed by other diagnostic cuts releasing fragments of the sizes predicted from the published restriction map of ricin A chain cDNA (Lamb et al., 1985), (lanes 3, 4 and 6). To determine whether or not the inserts had been ligated in frame with the Xenopus ATG codon it was considered that the most cost and time effective approach to screening the recombinants was to transcribe batches of the mini - DNA's using SP6 RNA polymerase and to translate them in a wheatgerm lysate.

9:2:3 Wheatgerm lysate translations.

Ricin A chain sequences in frame with the Xenopus ATG codon would appear as radiolabelled bands on an autoradiograph of a 10% polyacrylamide gel on which products were run. The Hind III / Cla I digest of figure 9.2.3. lane 6 shows a 200 - 300bp fragment was released. In a wild type sequence cloned similarly, the size of this fragment would be roughly 450 bp (see figure 8.2.3, lane 3 showing a Hind III / Cla I digest of ricin A chain cloned in pSP64ΔBam.). Therefore at most, 150bp have been removed, of this, nearly 100bp will be the ricin signal sequence. One would not, therefore, predict a great size difference between ricin A chain template without a signal sequence, (pSP64 BamΔA) and the deleted ricin A chains transcribed and translated in vitro and analysed by SDS - PAGE and

FIGURE 9.2.4 TRANSLATION OF MUTANT RICIN A CHAIN SEQUENCES IN WHEATGERM LYSATE.

Transcripts prepared in vitro encoded the following proteins synthesised in a wheatgerm lysate:

Lane 1 = ricin A chain; wild type. (pSP64 Bam A).

Lane 2 = ricin A chain from which the hexapeptide sharing homology with hamster EF 2 had been removed

Lane 3 = ricin A chain Ala 56

Lane 4 = ricin A chain Ala 48

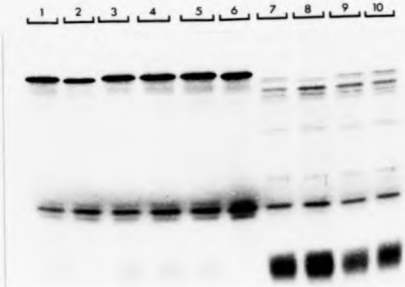
Lane 5 = ricin A chain Arg 56

Lane 6 = ricin A chain SHAAR

Lane 7 = ricin A chain from which the first 12 amino acids had been removed

Lane 8 = ricin A chain from which the first 9 amino acids had been removed

Lanes 9 and 10 are identical polypeptides and are identical to that in lane 7 (see fig 9.2.5).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10

autoradiography. Figure 9.2.4., lanes 7 - 10 represents four of these deleted ricin A chain templates translated in a wheatgerm which gave a signal on an autoradiograph previously (data not shown), of the size predicted from the restriction digests, compared with wild type and mutant A chains generated by site directed mutagenesis (lanes 1 - 6). It was evident that deletions were of roughly the same size and were not large. By taking this approach, RNA template of sufficient purity was available for the assessment of the toxicity of the polypeptide it encoded, without having to go through the procedure of producing large plasmid preps as template for RNA synthesis.

9:2:4. Plasmid sequencing.

In order to identify sequences deleted, plasmid of the same batch prepared for transcription was sequenced from an SP6 promoter - specific primer obtained from Boehringer, using the techniques described by Murphy and Kavanagh (1988), (see section 2:9:3 .). Sequencing reactions were run immediately on an 8% buffer gradient gel, dried and exposed to film. Figure 9.2.5. shows two of the recombinant sequences, Δ12 and Δ13 and indicates the region deleted and how this relates to the published ricin cDNA sequence. (Lamb et al., 1985). Remarkably, of the four in - frame deletions shown in lanes 7 to 10 of figure 9.2.4., Δ1, Δ12, Δ13 and Δ18, all but one of them, Δ12, were identical as confirmed by plasmid sequencing and both differ only by 6 bases.

FIGURE 9.2.5 PLASMID SEQUENCING.

Plasmid sequencing of four of the deletion mutants was carried out exactly as described in section 2:9:3. The deleted sequences are aligned alongside the wild type sequence for comparison. The junction of the fusion at the ATG codon is marked in.

G A T C
Δ12
WILD TYPE
Δ13
G A T C
START
START
Xenopus 5' untranslated
Hind III
Hind III
SIGNAL SEQUENCE

9.2.5. Assessment of biological activity of mutant ricin A chain polypeptides.

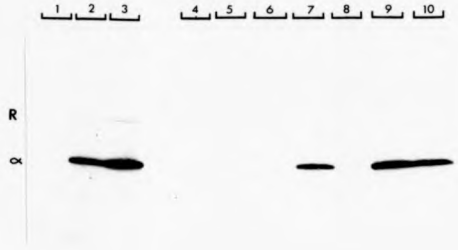
Equal quantities of RNA templates encoding polypeptides 1, 12, 13 and 18, were translated in a rabbit reticulocyte lysate for 1 hour and then 1ul of chicken lysozyme RNA was added for a further 30 min. Samples, (5ul), of each reaction were run on a 10% SDS - Polyacrylamide gel with other ricin A chain mutants generated as described in chapters 7 and 8 and wild type ricin A chain assessed similarly for comparison. The gel was dried down, fluorographed and autoradiographed and the result is shown in figure 9.2.6.

As would be predicted from the sequence data, 1, 13 and 18 exhibit the same activity in vitro and can be classed as the same mutation. The deletion of 12 amino acids had the effect of abolishing the catalytic activity of the protein; 1, 13 and 18 did not inhibit the subsequent translation of chicken lysozyme by rabbit reticulocyte ribosomes, which remain active. However, by delating 9 amino acids, (12), 3 amino acids less, the protein was fully functional and inhibited the subsequent translation of chicken lysozyme; this mutant polypeptide retained the ability to inactivate rabbit reticulocyte ribosomes. The ability of these mutants to depurinate 28S RNA was not assessed. The most likely explanation as to why the non - toxic mutants had been inactivated was that the deletion of 12 amino acids had caused a major conformational change in the protein, when expressed and that this caused the loss of activity. It was not considered likely that inactivation had been caused by the deletion of a region involved in determining the function of the protein. Presumably, the deletion of 9 amino acids did not cause a disruption of sufficient

FIGURE 9.2.6 ASSESSMENT OF ACTIVITY OF MUTANT RICIN A CHAINS

This figure shows the effect of wild type and mutant ricin A chain polypeptides on reticulocyte ribosomes. ^{35}S methionine supplemented reticulocyte lysates were allowed to translate, for 1 hour, transcripts encoding wild typee ricin A chain (lane 1), ricin B chain (lane 2), ricin A chain from which the hexapeptide sharing homology with EF 2 had been deleted (lane 3), mutant ricin A chain Ala 48 (lane 4), mutant Ala 56 (lane 5), mutant SEAAR (lane 6), mutant ricin A chain from which the first 12 amino acids had been removed (lanes 7, 9 and 10) and mutant ricin A chain from which the first 9 amino acids had been removed (lane 8). A second transcript encoding prepro alpha factor was added to each and translation continued for a further 30min, before the products were separated by SDS PAGE and visualised by autoradiography. R = ricin subunit bands

= prepro alpha factor.


1 2 3
4 5 6 7 8 9 10
R
α

magnitude to induce a conformational change and inactivate the protein. Whereas, when 12 amino acids were deleted, insufficient of the N - terminus of the protein remaining was remaining to permit its correct folding and subsequent activity. It would appear evident from this that the N - terminus of ricin A chain is crucial in determining the correct conformation of the active protein.

SECTION 9:3 SUMMARY.

The 5' terminus of ricin A chain cDNA was deleted using the enzyme Exonuclease III. Deleted fragments were forced cloned into a specially designed vector comprising an SP6 promoter, (for in vitro transcription of cloned sequence), downstream of which lay the 5' untranslated region of the Xenopus β globin gene, (to promote the stability of transcripts in vitro) and the Xenopus β globin gene ATG codon which would act as the initiating codon of any gene cloned in immediately adjacent to it. The blunt ended ricin A chain sequence was forced cloned into this vector by using the Pst_I site at the 3' end of the ricin A chain gene in the pGEM I multiple cloning site of pGEM IA, and the Pst i site of the pSP 64 polylinker region. The orientation of ligated inserts was tested by restriction digestion. Ricin A chain sequences in frame with the Xenopus ATG codon were identified by translation of SP6 generated template in a wheatgerm lysate. The identity of deleted sequences was confirmed by plasmid sequencing, directly from the same plasmid, using a primer which annealed specifically to the SP6 promoter. Two mutants were picked out for further analysis;

Δ12, lacking the 9 N - terminal amino acids,

and Δ13, lacking the 12 N - terminal amino acids.

The ability of these mutants to inactivate rabbit reticulocyte ribosomes was assessed using the same system as described in chapter 6. Mutant Δ12 retained full ribosome inactivating activity, whereas Δ13 had lost the ability to catalytically inactivate sensitive ribosomes.

The implications of these data are discussed fully in chapter 10 to allow comparisons to be drawn more conveniently between these and other ricin A chain mutants obtained in previous chapters tested in an identical fashion.

CHAPTER 10.

DISCUSSION.

SECTION 10:1. DISCUSSION.

10:1:1 General discussion.

In the present study a simple and sensitive system has been developed in which the expression and assessment of biological activity of ricin A chain are combined.(chapter 6). Conventionally, this would require the expression of ricin A chain in a heterologous microbial system such as E. coli (O'Hare et al., 1987) and the purification of the recombinant product prior to characterising its biological activity. The first important conclusion to be drawn, therefore, is that this system represents a considerable saving in time, effort and cost over conventional procedures. It was proven by numerous control experiments, (chapter 5), that the inactivation of the system (based on its ability to support subsequent translation) was due to the specific inactivation of its constituent ribosomes by recombinant ricin A chain expression and not due to any of the many other possible causes for lack of translation, including the generation of double-stranded RNA in the in vitro transcription reaction. The second major conclusion is that this system represents one of only two examples of the ability to assess the activity of protein expressed from in vitro synthesised RNA in a cell free lysate. (Glass et al., 1987).

The principle of the assay is to allow ribosomes which are sensitive to ricin A chain, namely rabbit reticulocyte ribosomes, to translate mRNA encoding recombinant A chain. Assuming that the A chain folds into a catalytically active conformation, the newly synthesised toxin should immediately begin to inactivate ribosomes and render them incapable of supporting subsequent translation. This is

feasible because ricin A chain is an extremely potent inhibitor of mammalian ribosomes (k_{cat} for ribosomes of 1500 min^{-1}, Olsnes *et al.*, 1975). It has been reported that a single A chain molecule located cytoplasmically is sufficient to inactivate all of the ribosomes given sufficient time. In this cell free system, because the ricin A chain is expressed and is immediately in contact with the ribosomes inactivation is rapid as demonstrated by the fact that there is very little accumulation of expressed A chain. The inactivation of the ribosomes by the newly-synthesised polypeptide is indicated by their failure to translate a second mRNA and was further supported by the demonstration that the 28S rRNA isolated from the same ribosomes was depurinated. The latter finding supported the theory that the inhibition of translation observed was due to the specific activity of ricin A chain and not due to inhibition caused by double - stranded RNA contamination, for example. This important finding demonstrates that not only does native ricin A chain possess RNA N - glycosidase activity, recombinant ricin A chain also has this function. In work carried out by Rupert Osborne, (May *et al.*, 1988, in press), primer extension was used to investigate in more detail, the precise position of the rRNA modification catalysed by ricin A chain synthesised *in vitro*. This confirmed that depurination was at exactly the same position as that reported by Endo and colleagues (1987) previously for rat liver 28S rRNA. Although this system only represents a qualitative assessment of the activity of cloned ribosome inactivating genes, it is assumed that it would function efficiently with transcripts encoding any cloned RIP. Currently many labs are attempting to clone RIP genes to provide material for the construction of recombinant immunotoxins. The use of this system would be a very suitable test to

rapidly establish the activity of such genes, before commiting time and effort to heterologous microbial expression.

10:1:2 **The relationship between inhibition of protein synthesis and 28S rRNA depurination.**

Similarly, the demonstration that ricin specifically depurinates 28S rRNA has been widely adopted as a diagnostic indication of RIP activity. However, it has not been proven conclusively that this is causitive of the inhibition of protein synthesis subsequently. An attempt was made to link these two properties of ricin functionally. (see section 6:2:3.). A roughly linear relationship was observed between the inhibition of protein synthesis in a yeast lysate by added native ricin A chain and its depurinating activity on the ribosomes in that system. This is consistent with the argument that depurination of the 28S rRNA causes the inhibition of protein synthesis; for every strand of RNA that is depurinated, one ribosome would lose the ability to support peptide chain elongation. The work of Moller and colleagues recently (Moazed *et al.*, 1988) strongly suggested, by analogy with the interaction of EF G and EF Tu and a loop of ribosomal RNA conserved between prokaryotes and eukaryotes, that depurination of the 28S rRNA prevents EF 1 and EF 2 binding to it and therefore directly inhibits protein synthesis. This is in accord with the bulk of early evidence proposing that competitive binding between ricin A chain and the elongation factors determined whether or not a ribosome was capable of supporting translation. (see, for example, Fernandez - Puentes *et al.*, 1976) What makes this line of argument even more attractive are the following observations:

1. The depurination of 28S rRNA is at position A_{4324} (Endo et al., 1987)

2. A_{4324} lies in a hairpin loop region strongly conserved between prokaryotes and eukaryotes (Veldman et al., 1981; Hogan et al., 1984)

3. Even though prokaryotes have this loop they are resistant to the depurinating activity of ricin (Endo et al., 1987), presumably the secondary structure of this region determined by the associated ribosomal proteins (which do differ between species) are important in determining this sensitivity.

4. Ricin A chain and alpha sarcin alter the conformation of rat 60S subunits in a specific way such that three ribosomal proteins; L3, L4 and L14 show changed reactivity with N - ethylmaleimide (Terao et al., 1986), L4 is thought to be located at the A site and L3 at the P site. Both proteins can be crosslinked to EF 2 (Uchiumi et al., 1986), are located at the boundary of the 60S and the 40S subunits and interact with mRNA (Tagahashi and Ogata, 1981 and 1985).

5. Competitive binding studies showed that pretreatment of ribosomes with EF 2 could prevent ricin A chain activity (Fernandez - Puentes et al., 1975 and 1976), ricin A chain reduces EF 1 and EF2 related GTPase activity (Benson et al., 1975)

6. High concentrations of Mg^{++} partially restored ribosome inactivation by ricin A chain (Cawley et al., 1979).

7. Evidence is accumulating to show that the RNA itself may have a possible catalytic role itself (Moore, 1988).

Taking into account all this accumulated evidence a possible mechanism of action for ricin A chain can be proposed:

The functioning of intact ribosomes during the process of peptide chain elongation.

The sequence of events described below is summarised in figure 10.1.

Figure 10.1 A: Elongation has commenced, amino acyl tRNA is bound to codon 2 on the mRNA in the A site of the ribosome. The P site has a peptidyl tRNA in place at codon 1.

Figure 10.1 B: EF 2 binds a GTP molecule to form the EF 2 - GTP complex. This complex binds to L4 associated with the A site and L3 associated with the P site, both located at the interface of the 60S and 40S subunits and interacting with the mRNA. Both L3 and L4 are located close together in the 60S subunit (Terao et al., 1988).

Figure 10.1 C: Translocation of the mRNA occurs moving codon 2 to the P site, vacated by the tRNA molecule previously bound through codon 1. Codon 3 moves into the vacated A site upon initiation of a new round of peptide chain elongation. The peptide chain has grown by 1 amino acid upon transfer of it from the tRNA bound to codon 1 to the codon bound to codon 2. The translocation event is associated with EF 2 release and GTP hydrolysis.

The functioning of intact ribosomes in the presence of ricin A chain.

The sequence of events described below is summarised in figure 10.2.

Figure 10.2. A: Ricin A chain binds to the ribosome with possible involvement of the ribosomal proteins, as a result of this positioning within the ribosome it is in the correct position to depurinate A_{4324}.

Figure 10.2. B: This may induce conformational changes in the ribosomal proteins

Figure 10.2. C: The binding of EF 2 is prevented due to the conformational changes within the ribosome, thus preventing the associated elongation events.

The end result is that translation is inhibited. The inhibition of protein synthesis by ricin A chain is known to be catalytic since ribosome inactivation is in a less than equimolar ratio. Indeed, it has been speculated that 1 molecule of ricin A chain could inactivate all of the ribosomes in a eukaryotic cell. Thus after depurinating the 28S rRNA, the ricin A chain molecule is released to move onto the next ribosome. For the inhibition of protein synthesis to be irreversible, the inactivation of the ribosome should be irreversible. However, Cawley and colleagues (Cawley et al., 1979) demonstrated that ribosomes could be protected from ricin A chain induced inactivation by treatment with 16mM Mg^{++} and furthermore, that upon inactivation, activity could be restored by treatment with the same concentration of Mg^{++}. They proposed that the effect of Mg^{++} was to induce conformational changes in the ribosome thus preventing ricin A chain binding. It is known that in the presence of non-physiologically high magnesium concentrations, the ribosome can still function despite the fact that the 28S rRNA had been depurinated. (Cawley et al., 1979). The depurination of 28S rRNA at A_{4324} must be highly specific in its effect on the inhibition of protein synthesis since it has been shown that the ribosomes of many organisms show RNA backbone cleavages which occur as normal physiological events during ribosome ageing, but are nevertheless still active in protein synthesis (Cahn et al., 1970; Ellis and Hartley, 1974). Indeed, during this present study, it was found, (M. R. Hartley, personal communication), that the ribosomal RNA of rabbit reticulocyte lysate treated with micrococcal nuclease was extensively degraded, yet the lysate could efficiently

FIGURE 10.1 THE ELONGATION STEPS OF TRANSLATION ON 80S RIBOSOMES

Schematic representation of events during peptide chain elongation:

 = EF 2

 = putative EF 2 receptors

 = tRNA molecule

P = P site

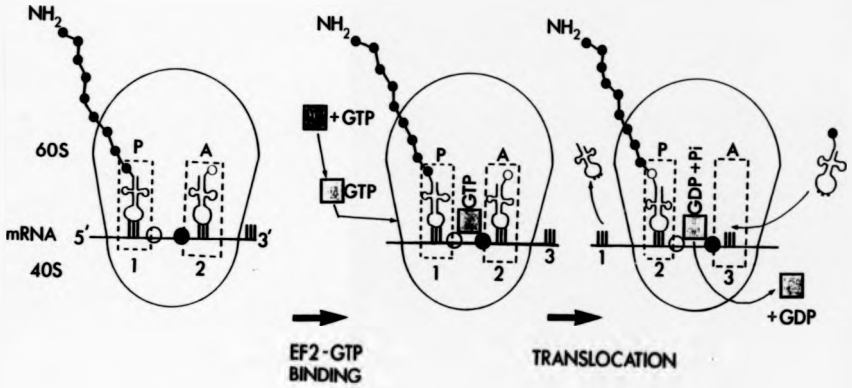
A = A site

 = growing polypeptide chain

1, 2 & 3 = consecutive codons on the mRNA molecule

FIGURE 10.2 THE EFFECT OF RICIN A CHAIN ON THE ELONGATION STAGE OF TRANSLATION ON 80S RIBOSOMES.

Labelling as above with ■ = ricin A chain.


NH2
60S
P
A
mRNA
5'
3'
40S
1
2
+GTP
GTP
GTP
3
EF2-GTP
BINDING
GDP +Pi
+GDP
TRANSLOCATION

GTP
A
P
GTP
A
P
A
P
mRNA

support translation. This would suggest that it is not just any modification of the 28S rRNA that would inhibit translation, and that it is not the depurination of 28S rRNA itself which prevents the ribosome functioning, but it is the conformational changes induced as a result of this very specific modification which are responsible for the inactivation. The partial rescue of ribosome function by Mg^{++} would support this theory. However, it should be pointed out that such non - physiological Mg^{++} concentrations would not be encountered in the cell and so in vivo ribosome inactivation would be irreversible. Cawley and his colleagues showed also that binding tRNA to the ribosomes prevented ricin A chain induced inhibition of protein synthesis (Cawley et al., 1979). This may explain the observation by Martin Hartley (personal communication), that free 60S subunits are preferentially modified by ricin A chain compared to ribosomes associated with polysomes where tRNA will be bound.

I must stress that although the model proposed above is supported by a certain amount of corroborative experimental evidence, it is largely conjecture.

If such a model is a valid assessment of the events occuring in a eukaryotic cell during the course of ricin A chain intoxication, then it is quite plausible to suggest the following: Subsequent to depurination of 28S rRNA conformational changes are induced in specific proteins. As a result of these conformational changes, ribosomes are no longer able to bind the EF 2 - GTP complex and protein synthesis is inhibited. Ribosomes are still able to initiate, yet can no longer support peptide chain elongation, (supported by the observation that the polysome profile of ricin treated cells remains intact, Martin Hartley, personal communication), subsequent treatment with magnesium may to some extent reverse these conformational changes and allow

translation to continue in vitro. Magnesium is known to have an effect on the conformation of RNA (Leaver, 1973) and is an important co - factor in nucleoside triphosphate hydrolysis reactions, which may be of significance in this respect. It would be the failure of ricin treated ribosomes to subsequently bind the EF 2 - GTP complex which ultimately determined their inability to support translation.

Thus the binding of ricin to the ribosomal proteins, which determine the secondary structure of the 28S rRNA in the target loop structure, must be of great importance to its subsequent depurinating activity. One could suggest that since binding of ricin and EF 2 on the ribosome is competitive, this could imply that they share a common binding site, since it is known that prebound EF 2 may protect ribosomes from the action of ricin. The protective effect of EF 2, unlike high Mg^{++}, would not be to induce conformational changes in the ribosomal proteins but would be a result of steric hinderence, physical blocking of the ribosome binding site thus blocking the entry of ricin A chain. If ricin A chain and EF 2 share a common ribosomal binding site, it would be quite feasible to propose that they should also share sequence homology, encoding a structure which could bind to a common structure on the ribosome.

10:1:3 The functional significance of amino acid homology between ricin A chain and hamster EF 2.

The cloning and DNA sequence of hamster EF 2 has recently been published. (Kohno et al., 1986; Nakanishi et al., 1988). The complete amino acid sequence has been deduced.

The N - terminal portion of the EF 2 shows homology with GTP binding proteins. The C - terminal half contains several regions which

have homology with its bacterial counterpart, EF G, suggesting that this region of evolution has been conserved during evolution and may interact with the ribosome. Similarly the region around A_{4324} on rat 28S rRNA has been conserved during evolution, EF 2 is thought to interact with this region. (Moazed *et al*., 1988). The possibility that ricin A chain and EF 2 compete for the same binding site on the 60S subunit prompted us to search for primary sequence homology between the two proteins.

Remarkably some homology was found between bases 223 - 240 on the ricin A chain sequence and bases 373 - 400 on the hamster EF 2 cDNA sequence. Furthermore, this sequence was shared by trichosanthin (Xuejun and Jiahuai, 1987), and abrin, (Funatsu *et al*., 1988). (see figures 1.5.1. and 7.1). The sequence of abrin was published subsequently to undertaking this work but the discussion of its homology is particularly relevant. When this sequence was deleted from the ricin A chain cDNA sequence and the resultant plasmid transcribed using SP6 RNA polymerase and expressed in the toxicity assessment system described in chapter 6 the polypeptide was found to be inactive. It showed no ability to inhibit the translation of an mRNA added subsequently to its translation and furthermore was unable to depurinate the 28S rRNA of the ribosomes which had translated it. The inactivation of this protein did not appear to be due to gross conformational changes indicating that the deletion in the DNA sequence was functionally significant. Since ricin A chain, trichosanthin and abrin and EF 2 share a common function; binding to ribosomes, it is possible that the region deleted in ricin A chain encodes a ribosome binding domain. This would aggree favourably with the model proposed above.

In an effort to substantiate that this region did in fact encode the ribosome binding domain of ricin A chain, the following experiment was planned: The corresponding region in hamster EF 2 cDNA would be deleted and the mutant polypeptide expressed in the toxicity assessment system described in chapter 6. Ricin A chain would then be expressed in the same reaction and its ability to depurinate the 28S rRNA of the ribosomes in that system assessed. A control experiment would be performed in which the wild - type hamster EF 2 cDNA was expressed similarly, followed by the expression of wild type ricin A chain. In the wild type EF 2 control experiment a situation similar to that described by Fernandez - Puentes et al., (1975), would be expected, where excess EF 2, prebound to the ribosomes would sterically hinder the access of ricin A chain expressed subsequently and so prevent ribosome inactivation which would be assessed by aniline incubation of the ribosomal RNA. Where mutant EF 2 was expressed initially, if the deleted region encoded a ribosome binding domain, no such protection would be expected and 28S rRNA depurination would be observed. Such a reciprocal deletion experiment would, I believe, indicate conclusively that the common region had the common function of ribosome binding. However, to my regret, the authors would not supply the DNA to perform the experiment. Other experiments were planned to substantiate the identity of the function of the DNA sequence described.

Expression of the mutant ricin A chain cDNA in E. coli would generate sufficient purified mutant polypeptide to perform ricin - ribosome binding studies. Hedblom and colleagues describe such an approach. (Hedblom et al., 1976). If the mutant ricin A chain showed a lower affinity for ribosomes compared to wild type recombinant A chain (O'Hare et al., 1987), this would be further evidence for a function in ribosome binding of the DNA sequence deleted. However

despite many attempts to express this mutant in E. coli. Mary O'Hare has not reported any success and so this work remains to be done. This failure to express this mutant in a heterologous expression system may indicate that the protein was aggregating out or was being specifically degraded.

10:1:4. Site directed mutagenesis and N - terminal deletion of ricin A chain cDNA.

Chemical modification studies have previously shown that the catalytic activity of ricin A chain (Watanabe and Funatsu, 1986) and the A chain - like protein gelonin from Gelonium multiflorum (Srinivasan et al., 1985), are dramatically reduced after modification of certain arginine residues. In the case of ricin A chain, the key arginine residues were located in the N - terminal peptic fragment. Three of these (Arg 29, 48 and 56) are conserved in trichosanthin suggesting they may be functionally important. Two of these (Arg 48 and 56) are also appropriately placed in the putative active site cleft described by Montfort and colleagues (Montfort et al., 1987). However, the single conversion of Arg 48 to Ala 48, or the conversion of Arg 56 to Ala 56, and the deletion of Arg 56, are without effect on the depurination activity of ricin A chain. It is possible that simultaneous substitution of several arginine residues may be necessary before toxicity is eliminated. The expression of the mutant polypeptides carrying the arginine mutations in vitro also depurinated the 28S rRNA of the ribosomes which translated them. Furthermore, primer extension analysis on the same RNA performed by Rupert Osborn

(May et al., 1988, in press) showed that like wild type ricin A chain expressed in vitro, the mutant polypeptides also modified the 28S rRNA at the same position. The demonstration that biologically active expressed toxin specifically depurinates 28S rRNA also supports the contention that inhibition of translation was not due to low levels of double stranded RNA contaminants which might have been generated during the transcription reaction. (Farrell et al., 1988).

Although the deletion of 15 bases from the ricin A chain cDNA encoding the pentapeptide SEAAR produced a polypeptide which retained full ribosome inactivating activity when expressed in the toxicity assessment system, the result was nonetheless intriguing. The glutamic acid residue (E), is reported to lie in the putative active site cleft (J.D. Robertus, personal communication) of ricin A chain. The sequence SEAAR is conserved between trichosanthin, ricin A chain and abrin. Sequence homologies are also shared in this region with barley protein synthesis inhibitor and the shiga - like toxins of E. coli. a glutamic acid residue being universally conserved. (see figure 10.3, below). Hovde and colleagues (Hovde et al., 1988) changed glutamic acid 167 of Shiga like toxin 1 to aspartic acid and found that this caused a thousand - fold reduction in the catalytic activity of the purified protein without causing great conformational changes. It was concluded that glutamic acid 167 is critical for the activity of of shiga - like toxin 1 A and may be located in the active site. Shiga like toxins have been shown to have exactly the same ribosomal RNA N - glycosidase activity as ricin A chain, (Endo et al., 1988). Therefore, why, if ricin A chain and Shiga - like toxin 1 A have the same mechanism of action and share homology in a region which is proposed as the active site of the latter, does a deletion in the same region of ricin A chain have no effect on its catalytic activity? The most

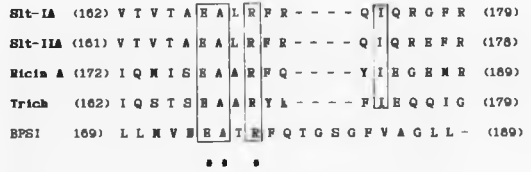


Figure 10.3. Alignment of amino acids in the A subunits of Shiga - like toxin I (Slt-IA) and Shiga - like toxin II (Slt-IIA), ricin A chain, trichosanthin, (Trich) and barley protein synthesis inhibitor II (BPSI). Conserved amino acids are enclosed in boxes. Asterisks indicate conserved residues in the cleft of the ricin A chain crystal structure. Numbers in parentheses refer to the positions of residues in the mature protein. Dashes indicate gaps introduced into the sequences to maximise alignments. This figure is taken from Hovde et al., 1988.

meaningful suggestion as to why this should be so is that the toxicity assessment system described in this present study would not detect such quantitative changes in the activity of the expressed protein. An important priority in subsequent work employing this technique would be to follow up in vitro studies with the expression of the mutant protein in E. coli and quantitative analysis of the kinetics of ribosome inactivation by the purified protein. Pseudomonas exotoxin A and Diphtheria toxin also reportedly have a glutamic acid at their active site. (Carrol and Collier, 1987), although it should be remembered that these toxins have a different mechanism of action.

Obviously, much work remains to be done in identifying the active site of ricin A chain. However, with the X - Ray structure available now and the increasing number of toxins sharing the same mechanism of action being cloned it could be envisaged realistically that future progress will be rapid. Furthermore, I believe that the experimental approach described in this thesis will play an important role in identifying the residues and domains essential for the biological activity of ricin A chain or any other cloned ribosome inactivating protein.

In a preliminary attempt to define an active A chain polypeptide of minimum size, the extent to which the N - terminus of ricin A chain can be deleted before activity was affected. The results suggest that there is little scope here: a series of proteins were generated with deletions at the N - terminus. Deleting the first nine residues from the N - terminus of the A chain appeared to have no effect on the activity of the polypeptide, whereas deleting the first twelve residues completely abolished activity. This could be due to the removal of amino acids important for activity, either in binding the toxin to the

ribosome or in the depurination reaction. I believe it to be due more simply to the result of erroneous folding. In contrast, it has been shown that the addition of extra residues to the N - terminus does not affect the biological activity of ricin A chain. Wild type A chain transcripts generated from pSP64 Bam A (chapter 7), encode a fusion protein encoding 14 additional residues at the N terminus and ricin A chain with a full 35 amino acid signal peptide are both fully active. There may be scope for limited C - terminal deletion, indeed Gary Clements, this lab, has reported that deletion of the hydrophobic C - terminus (29 residues) had no effect on the activity of the expressed protein when assessed in this system. It will be interesting to learn whether this mutant, when expressed in E. coli and purified will still retain the ability to be potentiated by ricin B chain. If it cannot, then this would pose serious problems for the generation of immunotoxins made up of ricin A chain with a deleted C - terminus.

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