POKEWEED ANTIVIRAL PROTEIN GENE: EXPRESSION AND APPLICATIONS

by

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ABSTRACT

Pokeweed antiviral protein (PAP) is a specific glycosidase which inactivates ribosomes by cleaving a single adenine at A^{4324} of 28S ribosomal RNA. Full-length PAP cDNA (PAP_f) was synthesized from pokeweed (*Phytolacca americana*) mRNA and was expressed in *E. coli*. The expressed PAP was biologically active.

In order to investigate the significance of the N- and C-terminal regions for toxicity, deletion mutagenesis on N- and C-terminal regions was performed. Results revealed that the N-terminal signal peptide (22 amino acids) but not the C-terminal region may be necessary for PAP gene expression.

To achieve a higher expression level of PAP, PAP_f was cloned in an expression vector containing a sequence (PL) derived from a polylinker instead of a Shine-Dalgarno (SD) sequence. Surprisingly, *E. coli* cells transformed with this vector produced over two times more PAP than with the vector containing the consensus SD sequence. Analysis of the sequence in front of the AUG codon revealed that the two boxes, ACCUACU (box I) and GAGUUAG (box II), were

complementary to 1434-1440 and 507-513 in the 16S rRNA, respectively. Mutational study indicates that PAP_f expression was dependent on the presence of both boxes. It is proposed that the two domains (507-513 and 1434-1440) in the 16S rRNA are organized in close proximity on the surface of the small ribosomal subunit and form a new mRNA binding site alternative to SD.

The PAP gene was transformed into tobacco plants and mammalian cells to test for antiviral resistance. The PAP gene with a deletion of 36 codons from the C-terminus was expressed in transgenic tobacco plants and conferred an antiviral activity as high as 99%. The PAP gene devoid of the sequence for the N-terminal signal ($PAP_{\Delta N}$) was also introduced into CD_4^+ cells and the transformed cells were subjected to HIV-1 infection. Preliminary results indicated that the $PAP_{\Delta N}$ gene did not render transformed $CD4^+$ cells resistant to HIV-1 infection. Since the $PAP_{\Delta N}$ gene was functional *in vitro*, it is suggested that the $PAP_{\Delta N}$ gene may be mutated after integration into the cellular genome perhaps due to toxicity to the $CD4^+$ cells.

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AIDS	acquired immunodeficiency syndrome
Ap	ampicillin
ATP	adenosine triphosphate
BAP	6-benzyl-aminopurine
BCIP	5-bromo-4-chloro-3-indolylphosphate p-toluidine salt
βME	β-mercaptoethanol
BSA	bovine serum albumin
Ci	curie
CIP	calf intestinal alkaline phosphatase
CP	capsid protein
CRS	*cis*-acting repressor sequence
ddNTP	2',3' dideoxyribonuleotide triphosphate
DEPC	diethylpyrocarbonate
DNase	deoxyribonuclease
dNTP	2'-deoxyribonucleotide triphosphate
ds	double-stranded
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
env	envelope
gag	group antigen
f	full-length
g	gravitational constant (9.8 m/s^2)
HIV-1	human immunodeficiency virus type 1
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan	kanamycin
LB	Luria-Bertani (medium)
LTR	long terminal repeat
mA	milliampere
MEM	Eagle's minimum essential medium
M-MuLV	Moloney murine leukemia virus
NAA	naphthaleneacetic acid
NBT	nitroblue tetrazolium
pH	$-\log[H^+]$
OD	optical density
ORF	open reading frame
PAP	pokeweed antiviral protein
PAP_f	full-length pokeweed antiviral protein gene
$PAP_{\Delta N}22$	N-terminal signal peptide (22 amino acid residues) deleted PAP gene
$PAP_{\Delta C}62$	C-terminus (62 amino acid residues) deleted PAP gene
PBS	phosphate-buffered saline

pBS$^+$	plasmid BlueScribe$^+$ (Stratagene Corp.)
pmole	picomole
PMSF	phenylmethylsulfonyl fluoride
pol	polymerase
Psi	packaging signal
PVX	potato virus X
PVY	potato virus Y
rev	regulator of expression of virion proteins
Ri	Rev-inducible sequence
RNAguard	RNase inhibitor (Pharmacia)
RNase	ribonuclease
RNasin	RNase inhibitor (Promega)
RRE	Rev-responsive element
RT	reverse transcriptase
S	Svedberg unit
S/D	Shine-Dalgarno (sequence -AAGGAGGU-)
ss	single-stranded
SDS	sodium dodecyl sulfate
TAR	*trans*-activation responsive element
tat	*trans*-activator of transcription
TB	terrific broth (medium)
TBE	1 X: 0.1M Tris, 0.1 M boric acid and 2 mM EDTA, pH 8.3
TBS	Tris-buffered saline
TER	SV40 polyadenylation terminator
TTBS	TBS containing 0.05% Tween 20
TE	10 mM Tris-HCl, pH 8.0, 1 mM EDTA
Ti	Tat-inducible promoter
TMV	tobacco mosaic virus
Tris	Tris(hydroxymethyl)-aminomethane
μCi	microcurie
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YT	yeast extract-bactotryptone (medium)

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INTRODUCTION

Pokeweed Antiviral Protein (PAP)

(A) Ribosome-inactivating proteins

Plants, like other living organisms, are endowed with a multitude of protein and peptide compounds. Most of them are products of the plant's metabolism and are necessary for the life of the plant. However, plants also synthesize a number of interesting proteins that have not been shown to have any vital function in the plant of their origin. Among the best examples of such proteins are the ribosome-inactivating proteins (RIPs) or antiviral proteins.

Studies on the distribution in the plant kingdom of species containing RIPs are still scarce (Merino *et al.*, 1990). Most species examined belong to the class of Angiospermae. RIPs have been identified and purified from both monocotyledonous and dicotyledonous plants, but no RIP has yet been purified from the class of Gymnospermae. So far, many RIPs including those from corn, barley, wheat and melon have been purified (Table 1).

RIPs are generally subdivided into two groups: type I and type II (Table 1). Type I RIPs are monomeric proteins and account for the majority of RIPs. This single chain is able to catalytically inactivate eukaryotic ribosomes (Nicholson and Blaustein, 1972; Baenzinger & Fiete, 1979). It does not possess an additional binding chain and therefore cannot adhere to and easily enter a cell. Examples of type I RIPs include dodecandrin (from *Phytolacca dodecandra*), dianthins (from *Dianthus caryophyllus*) and tritin isolated from wheat germ (*Triticum aestivum*). The first type I RIP to be identified and isolated was from pokeweed plants (Irvin, 1983). This RIP was studied for its inhibitory activity on plant viruses. Only after purification was its effect

Table 1. Ribosome Inactivating Proteins

Name	Location	yield (mg/100g)	Species	Ref.
Type I RIPs				
PAP	spring leaf	9.2	*Phytolacca americana*	*Irvin 1975*
PAP-II	summer leaf	3.6	*Phytolacca americana*	*Irvin et al.1980*
PAP-S	seeds	100-180	*Phytolacca americana*	Barbieri, 1982
Asparin 1	seeds	5.4	*Asparagus officinalis*	Bolognesi, 1990
Barley RIP	seeds	139	*Hordeum vulgare*	Coleman, 1982
Dianthin 30	leaves	2.0	*Dianthus caryophyllus*	Stirpe, 1981
Dodecandrin	leaves	3.4	*Phytolacca dodecandra*	Ready, 1984
Gelonin	seeds	250-300	*Gelonium multiflorum*	Stirpe, 1980a
MAP	roots	90	*Mirabilis jalapa*	Miyano, 1992
Maize RIP	seeds	12-20	*Zea Mays*	Walsh, 1991
Melonin	seeds		*Cucumis melo*	Ferreras, 1989
Saporin-S6	seeds	268-414	*Saponaria officinalis*	Barbieri,1987
Trichosan -thin	roots	140	*Trichosanthes kirilowii*	Maraganore, 1987
Tritin 32	seeds		*Triticum aesticum*	Reisbig, 1983
Type II RIPs				
Ricin	seeds	120	*Ricinus communis*	Olsnes, 1973
Abrin	seeds	100	*Abrus precatorius*	Lin, 1981
Ebulin	leaves	3.2	*Sambucus ebulus*	Girbes, 1993
Modeccin	roots	20-180	*Adenia (Modecca) digitata*	Olsnes, 1978
Viscumin	leaves	7	*Viscum album*	Stirpe, 1980b

on protein synthesis and on ribosomes discovered.

Type II RIPs are composed of a heterodimer of two proteins, an A (similar to Type I RIP) and a B chain, which are linked by a disulphide bond (Nicholson, *et al.* 1974). The B chain is a carbohydrate binding protein and binds to galactose residues on the cell membrane receptors and facilitates entry of the dimer into the cell through endocytosis (Nicholson *et al.*, 1975). Well-studied examples of this group include ricin (from *Ricinus communis*) and abrin (from *Abrus precatorius*).

Type I RIPs have the same activity, although at much higher concentrations, on rRNA purified from bacterial or mammalian sources and on synthetic oligonucleotides (Endo and Tsurugi, 1987; Endo *et al.*, 1987; Endo and Tsurugi, 1988). Still, their activity varies greatly among intact ribosomes from various organisms. Thus, type I RIPs are much more active on mammalian ribosomes (Endo and Tsurugi, 1987; 1988) and act to a variable extent on ribosomes from other organisms such as plants (including pokeweed plants), fungi, protists and bacteria (Bonness *et al.*, 1994). In contrast to type I RIPs, type II RIPs do not cleave a single adenine in the 23S rRNA and do not inactivate prokaryotic ribosomes possibly protected by ribosomal proteins (Chaddock *et al.*, 1996). Taken together, these results suggest that each RIP has a specific pattern of activity on targeted ribosomes of different genera, while ribosomes from each species have a specific spectrum of sensitivity to different RIPs.

(B) Pokeweed plants and their early history

Phytolacca americana, common name pokeweed (Fig.1), is a native species in North America. Of the 25 species in the genus *Phytolacca*, this is the only one represented in eastern

Fig.1. A pokeweed plant (*Phytolacca americana*)

North America (Britton and Brown, 1898). The range of pokeweed is centred north of the tropics and indigenous to Quebec, Maine, New York and Ontario to Minnesota, south to Florida and Texas. It is generally found in rich soils in waste ground, farm lots, around dwellings, fields, along roadsides and railroads etc. (Steyermark, 1964). Pokeweed is a large bushy herb, sometimes as much as 3.5 m tall, and its dark green, alternate leaves are up to 25 cm long. The flowers are numerous, small, white or greenish. The fruit is a 10-seeded, ink-juiced, shiny, purple berry (Kingsbury, 1964). Pokeweed has been of interest to humans as it has been used in medicine since ancient times. The Delaware Indians probably were the first to use it for cancers, the "itch", and rheumatism (Durant, 1976). It was employed through the 19th century (Lestrange, 1977) and farmers and dairymen still use a tincture of phytolacca for reducing caking and swelling of cows' udders (Spencer, 1968). However, little effort had been directed toward identifying the effective compounds present in pokeweed. Duggar and Armstrong (1925) first realized the pokeweed plant contained an antiviral agent. They discovered the antiviral activity after attempting to infect the pokeweed plant with tobacco mosaic virus (TMV), a task which they were unable to accomplish even after several attempts. They then tested the activity of the sap from the pokeweed plant with TMV and found that it reduced the infectivity of the virus in tobacco plants too, even after the sap was diluted several fold. Many other plant extracts were tested for antiviral activity but none were found to have the same level of potent antiviral inhibition. These authors concluded that the pokeweed plant contained a substance which was capable of inactivating the virus. The antiviral agent was first isolated from the plant sap by Kassanis and Kleczkowski (1948) and later it was found to be a protein (Wyatt and Shepherd, 1969).

So far, at least three different pokeweed antiviral proteins have been isolated. PAP-I or

PAP (hereafter referred to as "PAP" in this thesis) is found in spring leaves where it is located in the cell wall of leaf mesophyll cells (Ready *et al.*, 1986). As the season progresses into summer, the concentration of this protein declines and PAP-II accumulates in the leaves (Irvin *et al.*, 1980). The seeds of pokeweed also contain a ribosome inactivating protein, PAP-S (Barbieri *et al.*, 1982).

(C) Physicochemical properties

Interest in the RIPs including PAP was not revived until Lin *et al.* (1970) reported that RIPs were more toxic to tumorous than to normal cells. This observation was followed by a considerable amount of work on RIPs including PAP.

Unlike isolated A chain of type II RIPs, type I RIPs including PAP appear to be fairly resistant to a variety of physiological and chemical treatments. Thus, freeze-drying, repeated freezing and thawing, incubation at 37^0C for several hours, labelling with I^{125}, treating with proteolytic enzymes or with β-mercaptoethanol, change of pH and prolonged storage do not significantly affect the activity (Verma *et al.*, 1995). The molecular mass of PAP has been determined to be 29 kDa by polyacrylamide-gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE). The value obtained by complete sequencing (Chen *et al.*, 1991) is in agreement with that obtained with SDS-PAGE. The molecular masses of other RIPs are around 30 kDa, both for type I RIPs and the A chains of type II RIPs, and slightly higher for the B chains of type II RIPs (Table 2). The pI of all type I RIPs is invariably basic (Gelfi *et al.*, 1987).

Table 2. Properties of Some Ribosome-inactivating Proteins

Name	MW (Daltons)	Sugar content (%)	E280[a] (M)	pI[b]	Reference
Type I RIPs					
PAP	29,000	0	24,000	8.1	Irvin. 1983
PAP-II	30,000	0	26,700	8.3	Irvin, 1983
PAP-S	30,000	0	22,500	8.45	Irvin, 1983
Barley RIP	30,000		27,600		Leah, 1991
Dianthin 30	29,500	1.56			Stirpe, 1981
MAP	28,000		24,000		Habuka, 1989
Maize RIP	25,000		26,500		Walsh, 1991
Tritin	30,000				Roberts, 1979
Type II RIPs					
Ricin	63-66,000	5.5-6.2	77,900	7.1	Jansen, 1988
Ricin (A chain)	30,000	4.5	23,000	7.6	Jansen, 1988
Ricin (B chain)	34,000	6.4	50,700	5.5	Jansen, 1988
Abrin	60,100	5.5	70,700		Herrmann, 1981
Abrin (A chain)	31,000	Present			Herrmann, 1981
Abrin (B chain)	35,500	Present			Herrmann, 1981

[a] Extinction coefficient at 280 nm

[b] pH at which a protein charge is neutral

Most of the amino acid residues composing the N-terminal signal sequences are hydrophobic, a feature common to signal sequences in eukaryotes and prokaryotes (Kataoka *et al.*, 1991). The N-terminal amino acid composition is known for a number of RIPs. There is a higher degree of identity (83%) between PAP-I and dodecandrin, from an African relative of pokeweed, than between PAP-I and PAP-S (55%), which are located in the same plant but in different tissues. There is only 40% identity between PAP-I and PAP-II (Houston *et al.*, 1983). The difference between these two proteins is not the result of differential processing of the same polypeptide. Rather, it reflects the seasonal variation of gene expression as the pokeweed develops (Ready *et al.*, 1984a). The identity in amino acid sequence suggests that PAP-I and PAP-II are related and that they have diverged from a common ancestor (Houston *et al.*, 1983).

Comparison of amino acid sequences from the NH_2-terminal end of type I RIPs and the A chain of type II RIPs demonstrates similarity between these proteins, suggesting that they are structurally related. Five invariant amino acids, isoleucine, threonine, tyrosine, phenylalanine and alanine, are found in the first thirty amino acids and a large percentage of the amino acids are either identical or strongly conserved in these proteins. Studies of PAP, PAP-II, PAP-S dodecandrin and SO-6 from *Saponaria officinalis* found that the amino terminus of those RIPs begins with a stretch of hydrophobic amino acids (#1-18) followed by a region of amino acids with hydrophilic characteristics (#22-31). PAP-II shows variation due to a hydrophilic region found between amino acids 5 and 13 (Lappi *et al.*, 1985).

Complete amino acid sequences for several RIPs have been reported and variable identities found. PAP and PAP-S show 80% identity (Lin *et al.*, 1991; Kung *et al.*, 1990), but PAP has only a 31% identity with both ricin A-chain and trichosanthin and a 38% identity with Saponin,

the major species of RIP isolated from *Saponaria officinalis* (Lin *et al.*, 1991). Sequence comparison of 11 RIPs shows 12 absolutely conserved residues. These are Tyr16, Phe19, Arg24, Tyr72, Tyr123, Gly141, Ala164, Glu176, Ala177, Arg179, Glu206 and Try208 in PAP. Except Thr207 in mature PAP, all others contain Asn207. These conserved residues have been extensively studied in ricin and models for the active site are proposed mainly based on crystallographic structure (Katzin *et al.*, 1991; Robertus, 1991) and mutagenesis (Frankel *et al.*, 1990). Variants Tyr80-Phe, Tyr123-Phe, Glu177-Gln (Frankel *et al.*, 1990; Ready *et al.*, 1991) and Arg180-His (Frankel *et al.*, 1990) had a substantial reduction in activity (7 to 1000 fold). Variants Tyr72-Phe and Tyr18-Phe of *Mirabilis* antiviral protein (MAP) have substantially reduced inhibitory activity only against *E. coli* ribosomes (Habuka *et al.*, 1992), while the corresponding variants of the ricin A chain have reduced activity against mammalian ribosomes (Ready *et al.*, 1991). The fact that various mutations of Glu177 still produced ricin A-chain mutants that retained significant activity (1-5%) suggests that Glu208 could substitute for Glu177 once it had been removed. This hypothesis was supported by two studies. One of them showed that the double mutant Glu177Ala and Glu208Asp had an activity less than 0.1% of the wild type ricin A-chain (Frankel *et al.*, 1990) and the second (an X-ray analysis) revealed that Glu208 rotated into the vacancy which was originally occupied by Glu177 in mutant Glu177Ala (Kim and Robertus, 1992). Thus, the active site of ricin A-chain contains the invariant residues Tyr80, Tyr123, Gln173, Glu177, Arg180, Glu208, Asn209 and Try211. These residues correspond to residues Tyr72, Tyr123, Gln172, Glu176, Arg179, Glu206, Thr207 and Try208 in mature PAP.

Based on the crystal structure and site-directed mutagenesis studies of ricin A-chain, a mechanism for the N-glycosidase activity has been proposed (Ready *et al.*, 1991; Robertus, 1991).

The active site Arg180 interacts through ionic bonds with both the 3' and 5' phosphates of the adenine to be depurinated while Glu177 stabilizes an intermediate oxycarbonium ion generated by the leaving adenine. As the adenine leaves it takes on a negative charge that must be neutralized by protonation, which may be provided by multiple hydrogen bonding from Tyr80 and Tyr123.

Recently, the crystal structure of PAP has been resolved (Monzingo *et al.*, 1993). As might be expected, the overall structure of PAP resembles that of ricin A-chain in its general features and active site residues. The active site lies toward the centre of the molecule but is open for binding to the ribosome. It seems likely that the mechanism for the N-glycosidase activity of PAP closely resembles that postulated for the ricin A-chain since PAP has the same essential arginine and glutamic acid residues located in the same relative positions as those observed in ricin A-chain. One major difference between PAP and the ricin A-chain is the presence of two disulfide bonds between cysteine pairs 34/259 and 85/106 (mature PAP) or 56/281 and 107/128 (including the N-terminal signal peptide). These two disulphide bonds most likely contribute to the observed thermal stability of PAP. Removal of the single disulphide bond by genetic engineering in *Mirabilis* antiviral protein, not only reduced its heat stability but also increased its inhibitory activity against rabbit reticulocyte ribosomes more than 20-fold, possibly because the disulphide bond partially blocks the active site (Habuka *et al.*, 1991a).

Since these proteins are present at high concentrations in a wide variety of phylogenetically unrelated plants, it has been speculated that these proteins may play an important role in plant physiology. However, this role may vary throughout various plant species e.g., as a defense against viruses or other parasites (Stirpe *et al.*, 1992) or as a means of eliminating

altered ribosomes (Chaudhry *et al.*, 1994). While most RIPs are glycoproteins, RIPs derived from pokeweed plants contain no sugar moieties. Since some RIPs (gelonin, ricin A) can be partially deglycosylated without affecting their biological activity, and recombinant ricin A chain produced in *E. coli* is not glycosylated yet fully functional (Barbieri *et al.*, 1993), it has been speculated that the carbohydrate component does not play any major role in RIP activity.

(D) Enzymatic function: ribosome depurination

PAP has been known to inhibit viral transmission in plants since its discovery in 1925 by Duggar and Armstrong. It was not until 1973 that PAP was found to be an extremely potent inhibitor of protein synthesis in cell free extracts derived from rabbit reticulocytes (Obrig *et al.*, 1973). This observation led to the suggestion that one molecule of PAP could damage more than one ribosome and thus might have some enzymatic effect on the ribosomal structure. This hypothesis was confirmed by Dallal and Irvin (1978), who demonstrated that radioactively labelled PAP does not bind to ribosomes and that PAP-treated ribosomes isolated by ultracentrifugation were no longer functional for protein synthesis.

Two lines of evidence clearly suggested that PAP caused the inhibition of the elongation stage of protein synthesis. First, polyphenylalanine synthesis, which does not require the natural initiation process, was inhibited by PAP. Second, the extension of globin chains was inhibited. Further studies demonstrated that PAP had major effects on the ribosome-dependent activities of elongation factor (EF-1 and EF-2). Two lines of evidence indicated that EF-1 was inhibited by PAP: factor-dependent binding of aminoacyl-tRNA to the acceptor site of the ribosome decreased, as did ribosome-dependent hydrolysis of GTP mediated by this factor. The activities of EF-2 were

also affected by PAP modification of the ribosome: the formation of the EF-2-GDP-ribosome complex was inhibited and the ribosome-dependent hydrolysis of GTP was greatly stimulated on PAP-treated ribosomes (Obrig *et al.*, 1973). These results suggested that PAP damaged the ribosome at some site at which both EF-1 and EF-2 interacted during the elongation cycle, and PAP decreased their affinities for this site, and thus inhibited the elongation of proteins.

Additional studies indicated that the inhibition of enzymatic binding caused by PAP treatment of ribosomes was found to be overcome by increasing the EF-1 concentration. Since EF-1 is extremely abundant in eukaryotic cells, and is present in large molar excess relative to their ribosome and EF-2 content (Slobin, 1980), EF-1 is not a limiting factor for protein synthesis. The major effect of PAP on protein synthesis is the inhibition of the translocation reaction mediated by EF-2.

Other groups demonstrated that the functional damage of ribosomes caused by modification of rRNA by PAP was responsible for the inhibition of protein synthesis. Endo and co-workers (Endo and Tsurugi, 1987; Endo *et al.*, 1987) demonstrated that type I RIPs and the A chains of type II RIPs possess a unique RNA N-glycosidase activity and cleave the N-glycosidic bond of adenine^{4324} of 28S eukaryotic mammalian rRNA. This cleaved adenine residue is located in a highly conserved sequence of 5'-AGUACGAGAGGAAC-3' and in a stem-loop structure (Fig.2), which indicates that RIPs recognize this specific structure. The simple removal of one adenine base renders the 60S subunit of eukaryotic ribosomes unable to bind the elongation factor II with consequent arrest of protein synthesis.

Action of PAP on native pokeweed ribosomes is debatable. Early studies found they are completely resistant based on the result that no difference in protein yield was observed in the

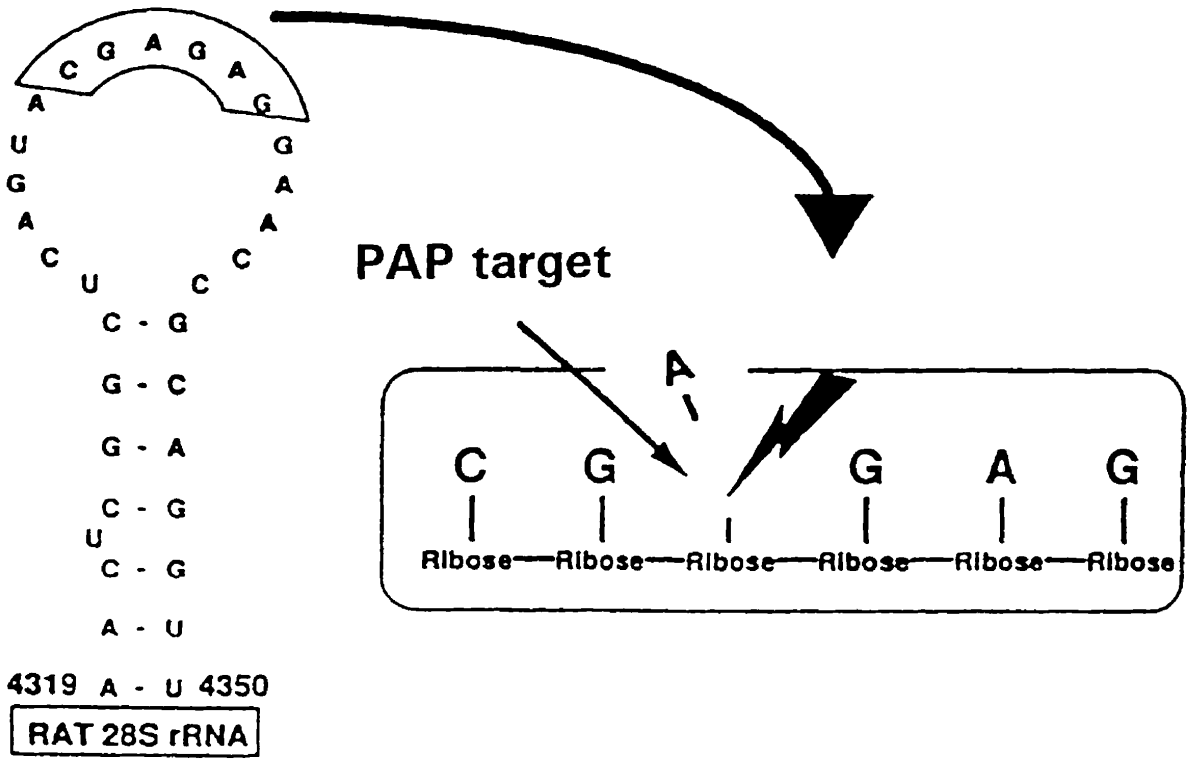


Fig.2. Mechanism of action of PAP. A specific N-glycosidase activity cleaves a single adenine base (A^{4324} in rat liver ribosomes) from the ribosomal RNA of the large subunit and causes complete inactivation of the ribosome. Adapted from Stirpe *et al.* (1992).

presence or absence of PAP in an *in vitro* translation assay (Owens *et al.*, 1973). However, this conclusion may be wrong because their pokeweed ribosomes exhibited low level of activity (1%) compared to the activities they obtained for wheat germ and cowpea ribosomes. Similar results were obtained by Battelli *et al.* (1984). It appears that ribosomes exhibiting high rates of protein synthesis are not available from pokeweed plants. This is explained by the fact that the PAP used in the experiments may be inactivated during the isolation procedures. A recent work found that PAP inactivated pokeweed ribosomes isolated from a cell suspension which does not express PAP (Bonness *et al.*, 1994). Thus, it seems PAP can inactivate its homologous ribosomes.

It was recently reported (Dore *et al.*, 1993; Chaddock *et al.*, 1994) that mutant forms of recombinant PAP had been produced, which, in contrast to native PAP, did not inhibit the growth of the host *E. coli*, but displayed catalytic activity when renatured from inclusion bodies and presented to eukaryotic ribosomes *in vitro*. However, the molecular basis of this difference in specificity is not known.

(E) Antiviral activity and its mechanisms

PAP (possibly other RIPs as well) has a broad antiviral spectrum. PAP has been shown to prevent infection by eight plant viruses: six RNA viruses, tobacco mosaic virus (Irvin *et al.*, 1980), southern bean mosaic virus (Wyatt and Shepherd, 1969), alfalfa mosaic virus, cucumber mosaic virus, potato virus X, potato virus Y; and two DNA viruses, African cassava mosaic virus (ssDNA) and cauliflower mosaic virus (ds DNA) (Chen *et al.*, 1991). All of the above can be readily quantitated by mixing with the compound and rubbing onto virus-susceptible leaves. As little as 50 nM of PAP is inhibitory. PAP has also been demonstrated to prevent infection of

animal viruses: herpes simplex virus-1 (Aron and Irvin, 1980), polio virus (Ussery *et al.*, 1977), influenza virus (Wyatt and Shepard, 1969) and human immunodeficiency virus (HIV) (Zarling *et al.*, 1990). The mechanism whereby PAP inhibits viral infections is still not clear. However, it seems that PAP does not directly inactivate the viruses since infectivity is inhibited if TMV is mixed with PAP before infection, but is recovered if the virus is separated from PAP (Ussery *et al.*, 1977; Foa-Tomasi *et al.*, 1982). In addition, PAP and other RIPs should be considered as a general inhibitor of viruses because PAP is active against both RNA and DNA, both enveloped and non-enveloped viruses. Further experiments suggest that the inhibition of infection can occur only at a very early stage of viral replication. This is based on the fact that PAP does not prevent attachment of virions to cells (Ussery *et al.*, 1977) but exerts antiviral activity only if applied to plants or cells before or at the same time of, but not after, virus infection (Chen *et al.*, 1991; Aron and Irvin, 1980). Therefore, the mechanism of viral inhibition is probably related to the ribosomal inactivating property of PAP.

Ready and co-workers suggest that PAP is synthesized in the cytoplasm, transported across the plasma membrane and trapped within the cell wall matrix (Ready *et al.*, 1986). Likely as in the case of ricin (Butterworth and Lord, 1983), the precursor form of PAP is processed within the cell wall to prevent self-inactivation. When the virus infects the plant cell, the wound process introduces PAP into the cytoplasm. PAP is then able to shut down the host ribosome and prevent viral propagation and spread.

It is possible that some other antiviral mechanisms are present, such as an inhibitor of viral enzymes. Lee-Huang *et al.* (1995) reported that two RIPs, MAP30 and GAP31 were capable of inhibiting infection of HIV-1 in T lymphocytes by inhibition of HIV-1 integrase. In this way,

the impediment of viral DNA integration may play a key role in the anti-HIV activity of these plant proteins. An alternative mechanism of antiviral activity was proposed to be caused by aggregation. It was reported that PAP and PAP-II were able to precipitate viruses so that the infectivity was decreased (Kumon *et al.*, 1990). A third mechanism for the inactivation is perhaps the inhibition of virus binding to the virus receptor although to date no such receptors have been physically isolated (Zipf, 1987; van Kammen *et al.*, 1961). Ragetli and Weintraub (1962) also suggested that the antiviral proteins may compete with viruses for the virus receptors, in other words, the presence of the antiviral proteins blocks the binding of viruses to their receptors. A final mechanism of viral inhibition is by induction of systemic resistance. Several plant extracts, when applied to test plants, have been reported to induce systemic resistance to viruses (Prasac *et al.* 1995; Verma *et al.*, 1996). The systemic resistance inducer has several properties similar to RIPs such as high pI (8.65), M_r 30 kDa, and a resistance to proteinases and heat. Treatment of plants with purified protein was shown to induce a very high level of systemic resistance against virus infection. The minimum time response for induction of systemic resistance was 5-30 min depending on the host. A proteinaceous virus inhibitory agent was subsequently isolated from the resistant tissue. An extract prepared from resistant leaves reduced the infectivity of added viruses, with an average reduction of 90% (Verma *et al.*, 1996).

(F) Heterologous expression of ribosome-inactivating protein genes

Much attention has been focused on the use of RIPs as antiviral agents. In contrast, relatively little is known about either the synthesis and targeting of RIPs or their role within plants. The biosynthesis of PAP by ribosomes must be compatible with the enzymatic activity

of these proteins that are capable of damaging the ribosomes. Three mechanisms appear to be involved in the "safe" synthesis of these proteins: 1) relative resistance of autonomous ribosomes to the actions of PAP; 2) synthesis of inactive pro-forms; 3) synthesis of pre-proforms with N-terminal signal sequences that direct the nascent peptide chain into the lumen of the endoplasmic reticulum before it is finally folded into the active configuration. Ricin biosynthesis has been studied in detail (Roberts and Lord, 1981). During ricin biosynthesis in *Ricinus* seeds, it is initially synthesized as a 576-amino acid pro-protein precursor with a 35-amino acid amino-terminal signal sequence and a 12-amino acid linker sequence between A chain and B chain. Upon synthesis, the nascent polypeptide is transported into the lumen of the rough endoplasmic reticulum where the amino-terminal leader sequence is removed by a signal peptidase yielding pro-ricin. Pro-ricin is glycosylated and the disulphide bonds established. From the endoplasmic reticulum, the pro-ricin is transported via the Golgi vesicles to the protein bodies. Here, pro-ricin is processed by an acid proteinase to the mature form.

One of the major problems encountered in working with RIPs is the expression of the gene product in an active form and in a quantity suitable for large scale exploitation. Ricin does not affect protein synthesis by ribosomes from *E. coli* or from rat liver mitochondria (Olsnes and Pihl, 1973; Greco *et al.*, 1974) but inhibits those from *Neurospora crassa* and from yeast mitochondria (Lugnier *et al.*, 1976). However, recently it was reported that type I RIPs can alter rRNA of *E. coli* ribosomes too (Hartley *et al.*, 1991; Prestle *et al.*, 1992). Several constructs containing sequences for RIPs could not be expressed at high levels in *E. coli* systems [10-30 μg/l of culture (Kataoka *et al.*, 1992; Shaw, *et al.*, 1991)] due to their toxicity to this translation system (Habuka *et al.*, 1989; Shaw *et al.*, 1991). Better yields were obtained by the use of

expression vectors under the control of the T7 promoter for trichosanthin (Zhu *et al.*, 1992) or the *tac* promoter for PAP (Kataoka *et al.*, 1993). Fully active recombinant PAP variants were purified from transformed cultures with yields varying from 1.74 to 5.55 mg/l depending upon the presence of a secretion *ompA* signal peptide. However, expression of fully functional ricin A chain or abrin in *E. coli* was obtained without toxicity problems (Kataoka *et al.*, 1992; Lamb *et al.*, 1985; Piatak *et al.*, 1988). In the case of ricin, the expressible plasmid directs the synthesis of a fusion protein containing the recombinant ricin A chain sequence and 10 extra amino-terminal residues under the control of a strong coliphage T5 promoter. This engineered product is fully active in inhibiting protein synthesis by a lysate of rabbit reticulocytes, and is capable of binding to ricin B chain to form a complex whose toxicity to cells is indistinguishable from that of the native ricin (Soler-Rodriguez *et al.*, 1992; Wu *et al.*, 1994).

(G) Applications of the PAP gene in agriculture: Development of transgenic plants resistant to viruses

Plant pathogens cause major losses in agriculture. Even now, after much research, crops still suffer, on average, 12% loss of yield worldwide (Moffat 1992). Moreover, modern agricultural practices have often exacerbated the disease problem so that some diseases may now be more damaging than they have ever been. In particular, certain agricultural practices designed to maximize short-term profitability have increased the likelihood of the disease. Such practices include the use of genetically uniform crop varieties over wide geographic areas, and the increased use of fertilizers and irrigation. In addition, modern agriculture uses more and more synthetic chemicals which kill plant pathogens for the protection of crops. Some of these

chemicals do not degrade easily in nature and therefore have been polluting farms, lands, rivers and eventually drinking water. In order to protect the environment from the pollution and reduce the losses to agriculture, many new approaches are being developed, and one approach is to develop disease-resistant plants.

The conventional method to develop disease-resistant plants is by breeding, but this normally takes many years. With the advent of genetic engineering, a novel approach has been studied and applied especially in virus resistance. It is known that expression of viral coat protein (CP) genes leading to the accumulation of high levels of CP in the transgenic plants provides protection against the virus from which the CP gene was obtained, and sometimes offers cross-protection against related viruses. For example, the accumulation of the CP of the common tobacco mosaic virus (TMV) strain U1 provides protection against TMV and tobacco mild green mosaic virus. However, that transgenic plant is not resistant to infection by other viruses (Stark and Beachy, 1989).

In order to provide a broader spectrum of resistance to different viruses, novel approaches are being developed. It has been known for many years that PAP, when mixed with plant viruses, can inhibit the mechanical transmission of the virus to host plants (Chen *et al.*, 1991). Direct application of antiviral agents to virus diseases in the field has been studied. Narayanaswami (1984) reported that if an antiviral agent from sorghum, *Basella alba*, is applied to plants prior to infection by tomato spotted wilt virus, the level of resistance in susceptible cultivars may be increased. Murthy and Nagarajan (1986) observed that a leaf extract of *Pelophorum ferrygineum* reduced the infection of TMV in tobacco both in the nursery and in the field after three sprays. Since several antiviral protein genes have been recently isolated and cloned, it is possible to

produce these proteins on a large scale with the use of genetic engineering. However, a more promising approach to virus control is to develop transgenic crops with an antiviral protein gene. Recent progress in the areas of *in vitro* tissue culture and genetic engineering allows the introduction of specific genes into the desired plant hosts. Lodge *et al.* (1993) reported that tobacco plants transformed with the full-length PAP gene, or a double mutant derivative of the full-length PAP gene, conferred resistance (21-100% depending on plane lines and plant viruses) to infection by different viruses. Thus, expression of PAP in transgenic plants offers the possibility of developing resistance to a broad spectrum of plant viruses by expression of a single gene.

(H) Applications of the PAP gene in medicine: Anti-HIV gene therapy

The most-studied application of RIPs is their use as immunotoxins, or conjugates with other suitable target-specific antibodies to eliminate in a selective manner any type of harmful cells. Immunotoxins have been proposed to attempt a cure for acquired immunodeficiency syndrome (AIDS) patients. AIDS is a disease caused by the human immunodeficiency virus type 1 (HIV-1) (Fig.3), and characterized by a slow, progressive impairment of the immune system, the appearance of opportunistic infections and encephalopathy. Since AIDS was first recognized in 1981 (Gottlieb *et al.*, 1981), it has claimed the lives of approximately 150,000 victims in the United States up to late 1992 (McCutchan, 1993), and 12 million were infected by HIV worldwide (Merlens *et al.*, 1994). It is estimated that a total of 37-93 million people from the beginning of the epidemic until the year 2000 will be infected (Janeway and Travers, 1996).

Although two immunotoxins (PAP and PAP-S conjugated with anti-HIV-1 envelope

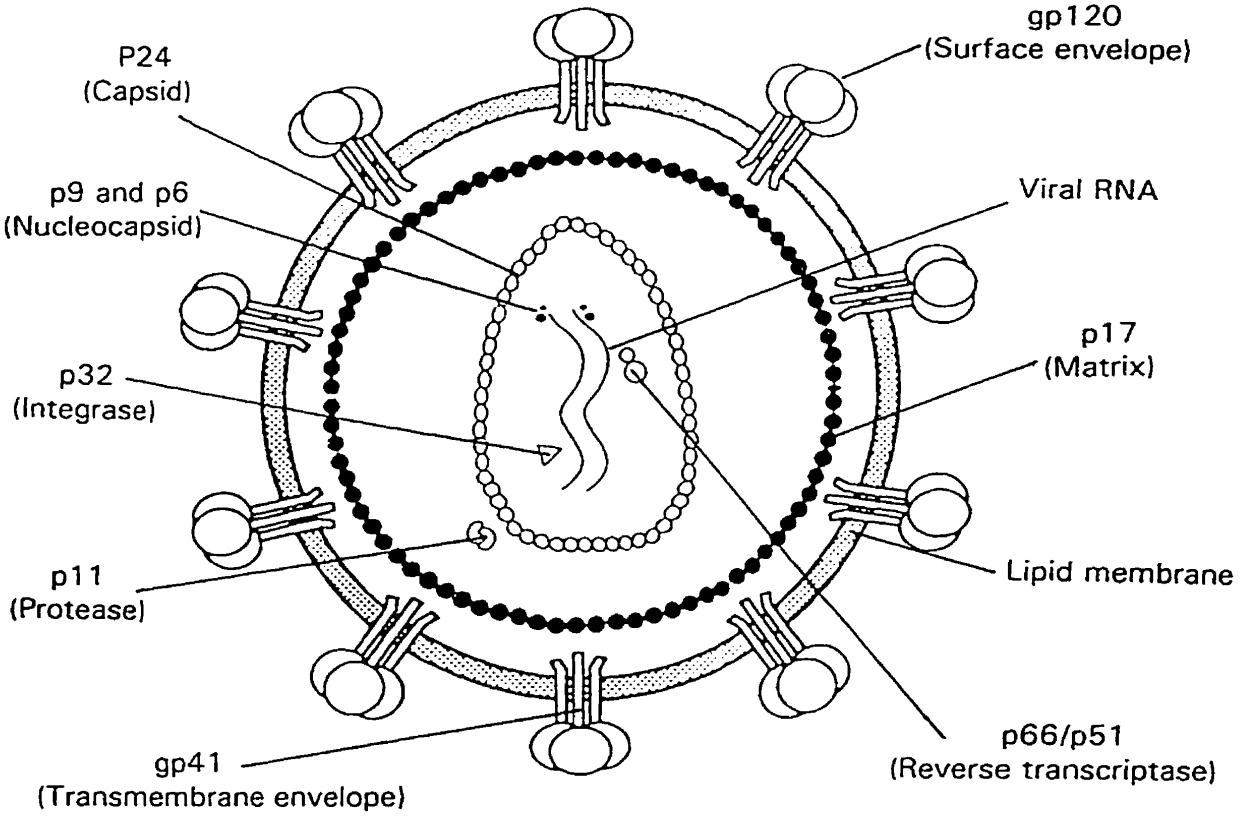


Fig.3. Structure of a HIV-1 virion. HIV-1 particles are spherical, about 110 nm in diameter, and consist of a lipid bilayer membrane surrounding a conical nucleocapsid. Within the nucleocapsid are two identical strands of RNA, which encode at least 17 different proteins. [Adapted from Mann (1996); Luciw and Shacklett (1993)].

protein gp120 antibodies, respectively) were reported to inhibit replication of HIV (Zarling *et al.*, 1990; Kim *et al.*, 1990), gp120 is highly variable and heterogeneous and it can be shed from infected cells and bind to uninfected CD_4^+ cells as well. In addition to the immunotoxins, many other drugs, mainly nucleoside analogs (Kent and Rud, 1996) and HIV protease inhibitor (Lea and Faulds, 1996), are currently being used to treat patients infected with HIV but easy and quick emergence of resistance to drugs may ultimately limit the usefulness of such drugs even when given a combined therapy (Katz and Skalka, 1994; Jadhav *et al.*, 1997). Therefore, novel approaches to cure AIDS are being sought.

Gene therapy has recently been considered as a promising approach to treat human immunodeficiency virus (HIV) infection (Sarver and Rossi, 1993; Dropulic and Jean, 1994). One of the gene therapy strategies is called intracellular immunization, which involves the genetic modification of cells to render them incapable of supporting viral production. This is achieved by placing in potential target cells a "suicide " gene under the control of HIV regulatory sequences (Harrison *et al.*, 1991). The *PAP* gene provides an excellent choice for this purpose.

(1) Life cycle of HIV-1 infection

The life cycle of HIV-1 can be divided into two major phases: early and late (Fig.4). In the first phase, virus particles attach to the receptor, $CD4^+$, via the envelope glycoproteins. Attached viruses penetrate the host cell membrane by a mechanism that is not fully understood but is believed to involve receptor-mediated endocytosis (Haseltine, 1990). The virus then uncoats and the contents including its RNA genome, virus-specific reverse transcriptase, integrase etc. are released. The single stranded RNA is reverse-transcribed into double-stranded DNA and the linear

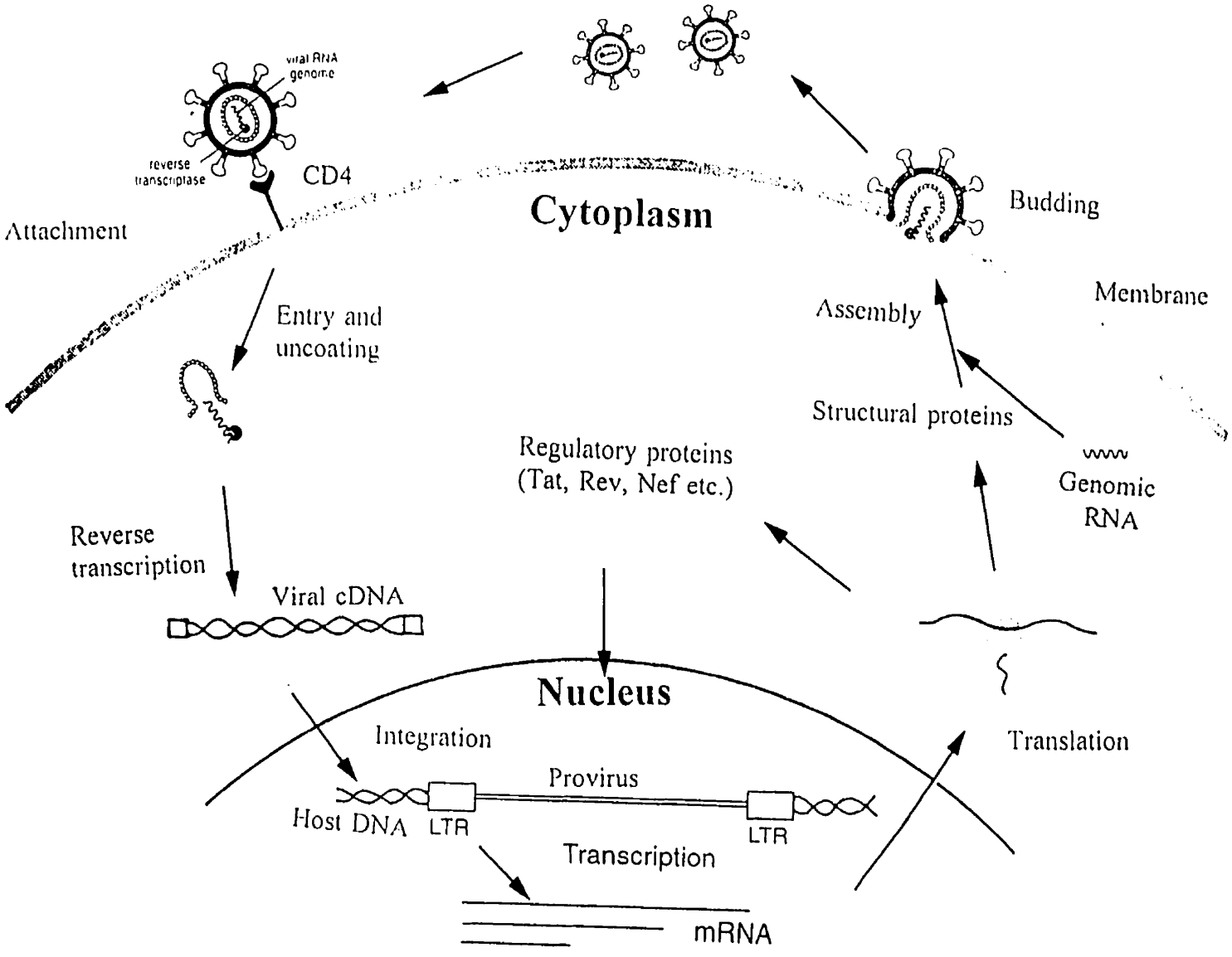


Fig.4. Life cycle of HIV-1. Modified from Janeway and Travers (1996), Luciw and Shaklett (1993).

form is transported into the nucleus and integrated into the host DNA with the aid of the integrase. In the integrated state, the virus can remain transcriptionally inactive or latent, and the viral genetic information remains in the cell as long as it survives and replicates every time the infected cell DNA is replicated.

In the second phase of the life cycle, the integrated provirus is transcribed by a cellular RNA polymerase into viral RNA. The mRNAs are translated on the cellular ribosomes, producing the structural and accessory gene products. The structural proteins make their way to the plasma membrane where they are assembled, incorporating the full-length transcript into virus particles. The virus particles are released from the cell by budding, then go on to infect other cells, thus re-initiating the replication cycle.

(2) Genome organization

The nucleotide sequences of many HIV-1 isolates have been determined and the HIV-1 genome was found to be approximately 9200 bp (Mann, 1996). HIV-1 genome contains the obligatory *gag*, *pol*, and *env* genes, flanked on the 5' and 3' ends by the Long Terminal Repeats (LTRs) (Fig.5). The *gag* and *pol* genes are in different open reading frames and overlap each other. The *env* gene is found at the 3' end of the genome separated from the *pol* sequences by the complex "central region". In the central region are a series of five ORFs that have been identified as *vif*, *tat* exon 1, *rev* exon 1, *vpr* and *vpu*. The *tat* and *rev* genes comprise two exons; the second exons of *tat* and *rev* are found in the 3' end of the *env* region but are in different reading frames from *env* and each other. The proviral DNA contains two copies of the viral LTR, one at each end of the genome. In the 5' to 3' direction, the LTR can be dissected into U3, R,

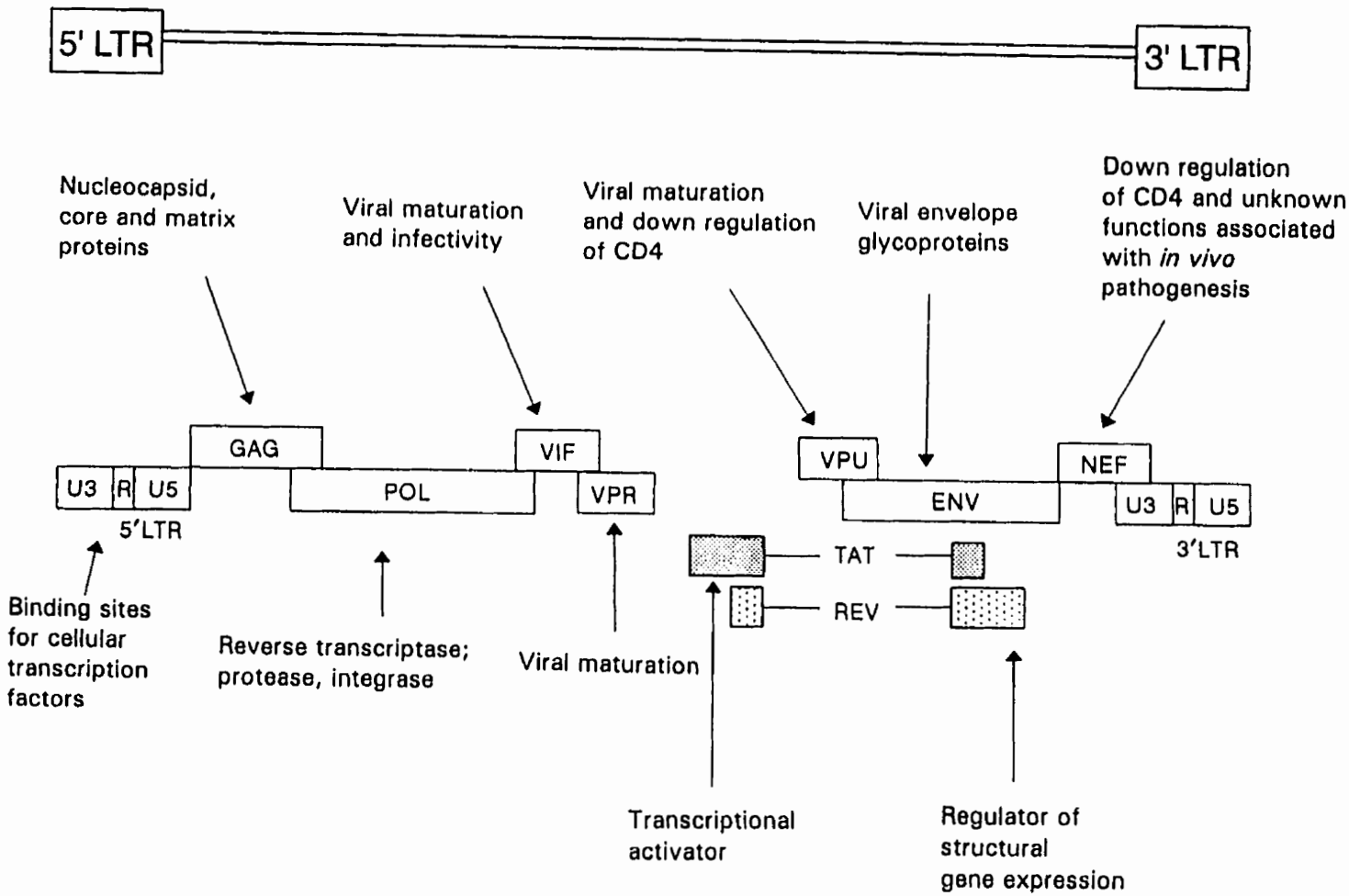


Fig.5. Genomic organization of HIV-1. Location of the genes and functions of their protein products are shown. [Adapted from Janeway and Travers (1993), Mann (1996)].

and U5 regions. The U3 sequences are derived from the 3' end and U5 sequences from the 5' end of the viral RNA; the R sequences are present at both ends. LTR contains a number of *cis*-acting sequences whose functions are to initiate, enhance and terminate transcription and integration.

(3) Components (Tat, TAR and Rev) involved in regulation of transcription and translation of HIV-1 proteins

(i) Tat (*Trans*-activator of transcription)

HIV gene expression is controlled by the binding of viral regulatory proteins to specific RNA target sequences. HIV Tat regulatory protein is the first activator that is known to stimulate transcriptional elongation (Fig.6). In the absence of Tat, RNA polymerase is halted and transcription is arrested near the viral promoter, preventing viral replication. Tat relieves this transcriptional block and stabilizes RNA polymerase downstream of the promoter (Jones and Peterlin, 1994). Tat is a 16 kDa (86 amino acids) viral protein and is produced early during the virus life cycle from multi-spliced, 2-kb mRNAs (Liem *et al.* 1993). It activates transcription from the 5' viral LTR, increasing the steady-state level of viral RNA in infected cells (Sodroski *et al.*, 1985). Tat also appears to enhance translation of viral mRNAs through interaction with viral RNA at the "TAR" (*trans*-activator responsive element) site, a region of conserved secondary structure at the 5' end of all viral RNAs. Tat protein has been shown to contain three functional domains. The acidic amino-terminal region forms an amphipathic alpha-helix and is required for full *trans*-activation (Rappaport *et al.*, 1989). A second domain is a cysteine-rich region which is involved in the formation of metal-linked Tat homodimers (Frankel *et al.*, 1988). This domain has been shown to be required for maintenance of a replication-competent phenotype

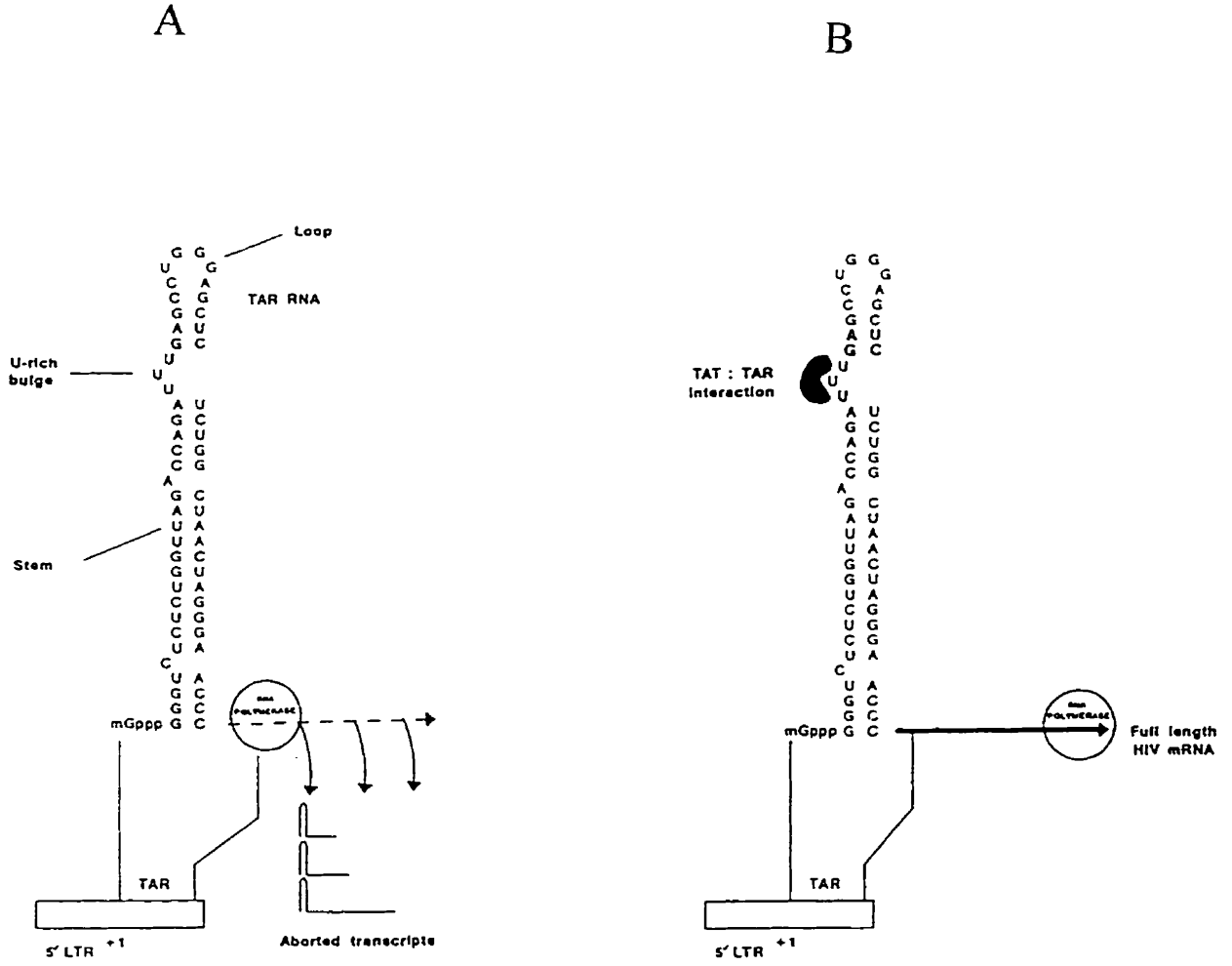


Fig.6. Effect of *trans*-activator Tat on transcription. Transcription of the first 60 nucleotides of the HIV-1 mRNA produces the TAR hairpin consisting of a stem-loop structure interrupted by a U-rich bulge. In the absence of Tat the cellular RNA polymerase easily falls away resulting in aborted HIV transcription. However, in the presence of Tat an interaction between Tat and the U-rich bulge improves the RNA polymerase function and full-length HIV RNA is generated. [Adapted from Mann (1996)].

(Rice and Carlotti, 1990). Another domain, rich in basic amino acids, is located in the carboxyl portion of the protein and is required for localization of Tat to the nucleoli of infected cells. This region is also responsible for the binding of Tat protein to the TAR (Ruben *et al.*, 1989). A trans-dominant *tat* mutant is capable of inhibiting *tat*-induced gene expression from the 5' LTR and conferring anti-HIV-1 resistance (Liem *et al.*, 1993). The inhibitory action of defective regulatory proteins suggests a role for them in antiviral strategies.

(ii) TAR (*Trans*-activator responsive region)

HIV-1 gene expression is regulated by the interaction of cellular factors with distinct regulatory elements in the HIV-1 LTR. One of these regulatory regions, designated TAR, is critical for transcriptional activation by the *trans*-activator protein Tat. TAR is a 59 nt RNA sequence that is located within the 5' noncoding region of all HIV-1 mRNAs (Selby *et al.*, 1989). The TAR domain, minimally required for Tat response, includes the Tat binding pyrimidine bulge, the TAR RNA upper stem, and the loop sequences. The secondary structure of TAR forms a stable stem-loop structure that contains several critical regions. Extensive mutagenesis, chemical probing and peptide binding studies (Churcher *et al.*, 1993; Hamy *et al.*, 1993) have defined the key regions required for TAR recognition by Tat and have shown that the Tat binding site surrounds a UCU bulge located near the apex of TAR (Delling, 1992). Tat interacts with the first uridine base (U23) of the bulge, while the other residues in the bulge act mainly as spacers and may be replaced by other nucleotides or even by non-nucleotide linkers (Churcher *et al.*, 1993). Tat recognition requires two base-pairs (G^{26}-C^{39} and A^{27}-U^{38}) in the stem above the U-rich bulge, (Churcher *et al.*, 1993; Delling *et al.*, 1992). Two base pairs (A^{22}-U^{40} and G^{21}-C^{41}) below the

bulge also make significant contributions to Tat binding. In addition, the preservation of the structural integrity of the lower stem structure of TAR plays an important role in viral growth, presumably by binding to specific host proteins that stabilize Tat-TAR interactions (Rounseville *et al.*, 1996).

(iii) Rev (Regulator of expression of virion proteins)

Rev is a 19 kDa (116 amino acids) viral, nuclear localized protein and is produced early during virus life cycles from multiply-spliced, 2 kb mRNAs (Sodroski *et al.*, 1986; Cullen *et al.*, 1988). Rev protein enhances production of viral structural proteins by facilitating the transport of unspliced and singly spliced viral RNAs from the nucleus to the cytoplasm (Felber *et al*, 1989; Emerman *et al.*, 1989), thus promoting the transition from the early to late phase of the HIV-1 life cycle (Cullen, 1991). The functional expression of Rev is essential for HIV-1 structural gene expression. The Rev protein interacts with a structured RNA target (RRE, Rev-Responsive Element) present in unspliced or incompletely spliced messages (Cullen, 1991). This interaction has been shown to override the inhibitory action of *cis*-acting repressor sequences (CRS) present within the HIV-1 *gag*, *pol*, and *env* coding regions (Cullen, 1991). Like Tat, Rev contains a basic domain that is required for both nuclear localization of the protein and RRE binding (Malim, 1989). In addition, a Leu-rich region toward the carboxyl terminus has also been shown to be important for Rev activity since substitution mutants were able to bind RNA but were not functional. This region may also play a role in the interaction of cellular proteins involved in the processing and/or transport of viral structural proteins.

In this study, a gene delivery system (retroviral vector) based on Moloney murine

leukaemia virus is used and the PAP gene is constructed into the retroviral vector to be controlled by HIV regulatory proteins Tat and Rev. Consequently, PAP will be expressed only in HIV infected cells. PAP gene expression will be switched on by HIV regulatory proteins Tat and Rev during the early phase of HIV replicative cycles. In HIV infected cells, PAP will induce cell death before the production of newly synthesized viral particles, preventing virus spread (Fig.7). As a long term goal, the bone marrow of AIDS patients will be treated with a mouse leukaemia virus-based retroviral vector. After transplantation and differentiation, PAP expression will take place in HIV-infected cells only and will result in cell death.

High Level Expression of PAP in *E. coli*: Problems and Solutions

PAP, and particularly its conjugates with specific antibodies (immunotoxins), had been demonstrated to be very promising in chemotherapy against various types of cancer (Frankel *et al.*, 1995) and viral infections (Irvin and Uckun, 1992). However, adequate expression of PAP in both *E. coli* and yeast systems has long been a problem and better expression systems are highly desired. It is known that protein production could be both transcriptionally and translationally regulated. A novel translational initiator may provide a better yield of protein production. Moreover, since the interaction of mRNA and ribosomes in protein biosynthesis is not clear, studies in this field may also answer some basic questions regarding the location of the mRNA on the ribosome and with which components it interacts.

Ribosomes are large ribonucleoprotein particles. *E. coli* ribosomes consist of a large 50S subunit (23S and 5S rRNAs + 34 ribosomal proteins) and a small 30S subunit (16S rRNA + 21 ribosomal proteins). The structure of ribosomal RNA (rRNA) and its role in protein biosynthesis

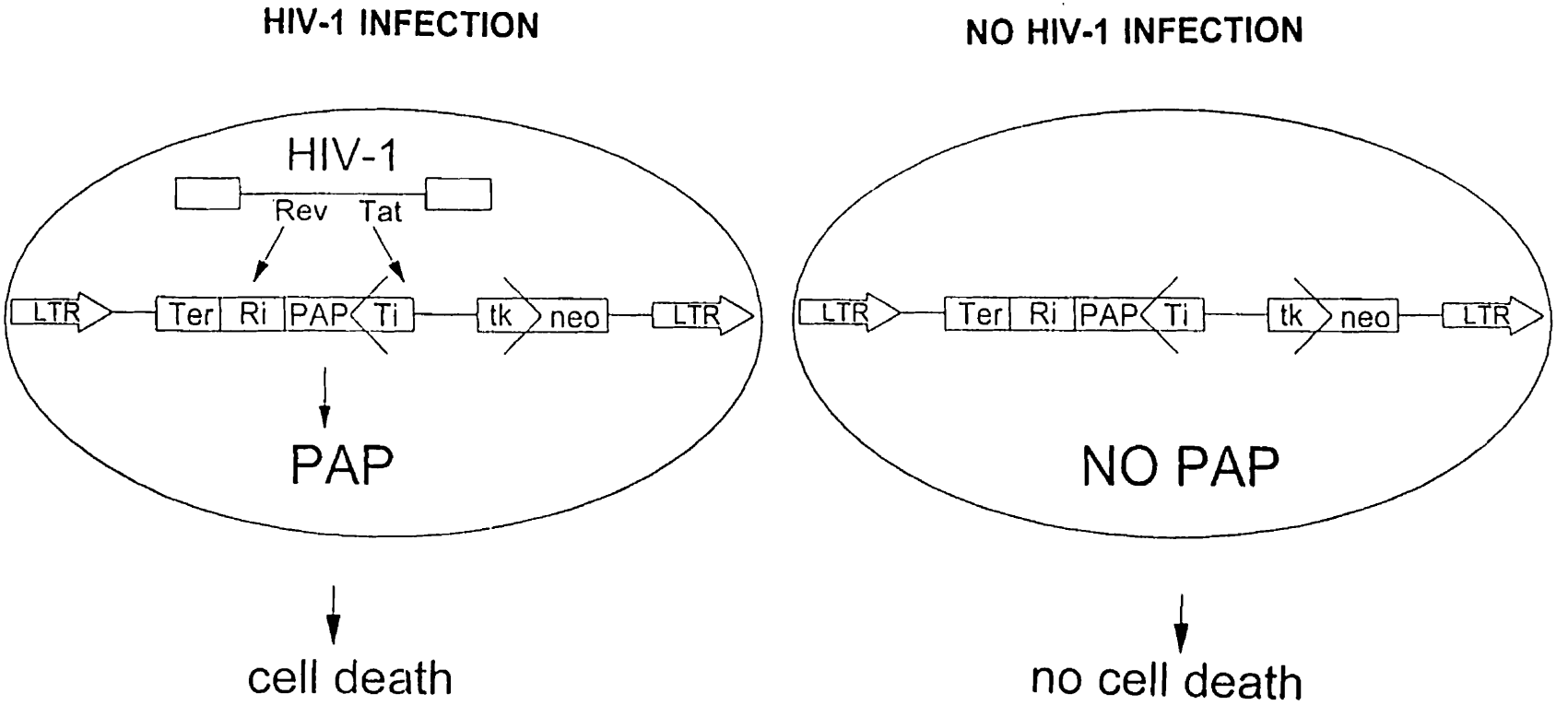


Fig.7. Selective killing of HIV-infected cells. The two ovals represent two CD4$^+$ lymphocyte cells containing integrated PAP gene and its inducible regulatory genes (Ti and Ri). Without HIV-1 infection, PAP gene remains silent and the cell (right oval) survives and propagates. If the cell is infected by HIV-1, the silent PAP gene is now activated by HIV-1 early proteins, Tat and Rev. Thus, the cell (left oval) is killed, preventing HIV-1 replication and spread. LTR, long terminal repeat; *neo*, neomycin phosphatransferase gene (conferring G418 resistance); PAP, pokeweed antiviral protein gene; Rev, regulator of expression of virion proteins; Ri, Rev inducible sequence; Tat, *trans*-activator of transcription; Ter, transcriptional terminator; tk, thymidine kinase promoter; Ti, Tat inducible promoter.

has been the subject of intense investigation for many years, and interest in this field has increased significantly. Most ribosomal proteins are now considered to have an auxiliary role in translation, acting to stabilize and modulate the structure of the functionally important rRNA domain (Santer and Dahlberg, 1995). It is known that rRNA structure is very delicate and minor changes result in the loss of biological activity. For example, mutation at positions 13 or 914 of *E. coli* 16S rRNA exerted pleiotropic effects on protein synthesis by interfering with the binding of streptomycin (a translational miscoding drug) to the ribosomes (Brakier-Gingras, *et al.*, 1995). Due to improved technologies such as cross-linking, footprinting, intra-RNA, RNA-protein interactions and DNA-hybridization electron microscopy, assays are now available for detecting the locations of ribosomal components (Brimacombe, 1995; Heilek and Noller, 1996; Lodish *et al.*, 1995; Oakes and Lake 1990). Great progress has been made in unraveling the overall structure of bacterial ribosomes and in identifying reactive sites in the rRNA that bind specific proteins, mRNA and tRNA. Moreover, a "head and body" model for the 30S subunit is generally accepted (Brimacombe, 1995; Malhotra and Harvey, 1994). Thus, helices 1-18 comprise the main part of the body of the subunit, helices 22-24 lie in the lateral protuberance or platform, and helices 28-43 comprise the head. The pseudoknot helices 1 and 2 are central to the structure, since the 5'-domain (helices 1-18), the central domain (helices 19-27), the 3'-domain (helices 28-43) and the 3'-minor domain (helices 44-45) all radiate out from this feature (Fig.8). It should be noted that the head and the body of the 30S subunit are connected only via helix 28, which implies that the neck of the subunit must be very narrow.

It is generally accepted that efficient translation of bacterial mRNAs depends on the presence of a Shine-Dalgarno (SD) sequence 5' to the initiation codon. The vast majority of *E.*

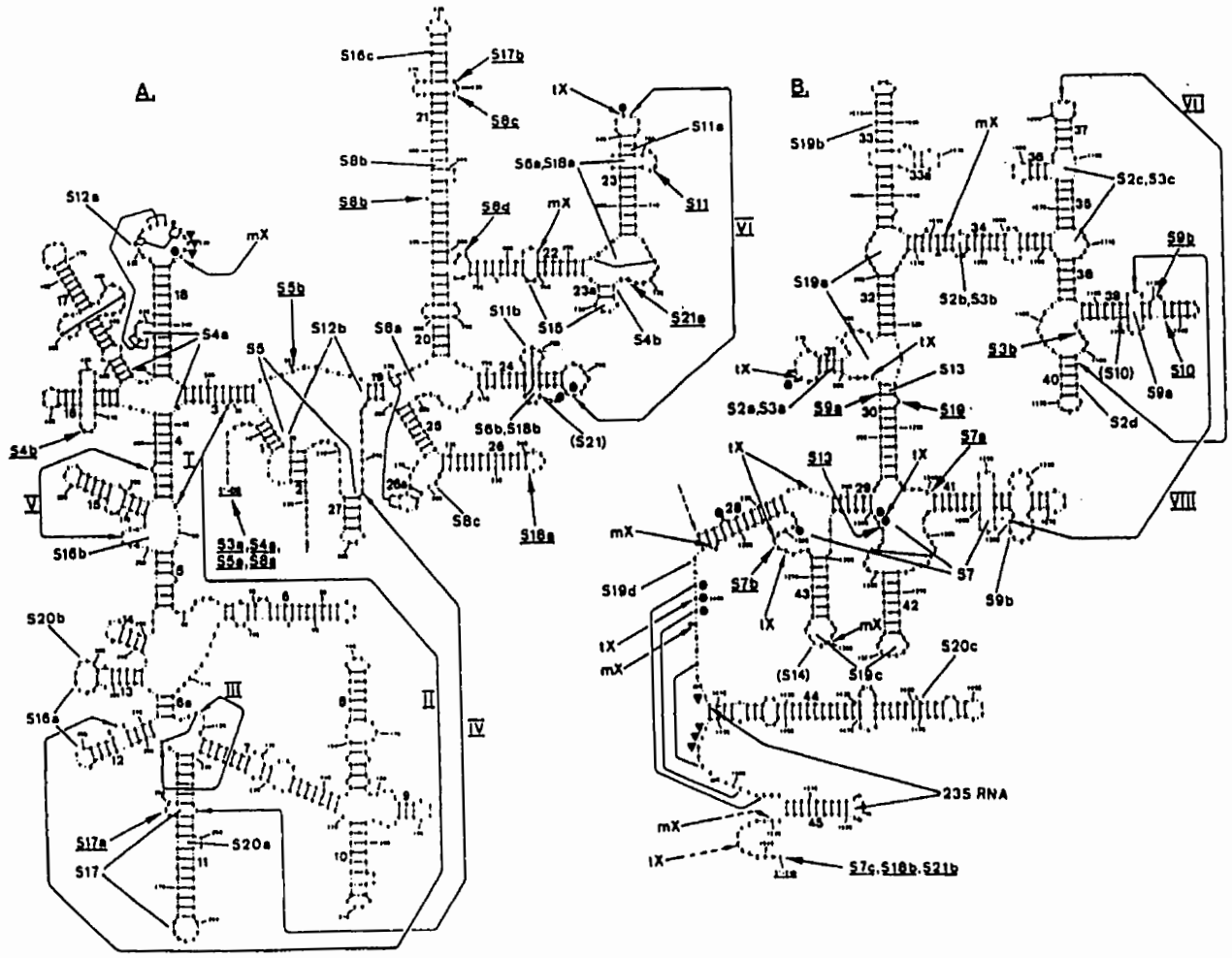


Fig.8. Topographic information in the complete 16S rRNA molecule. The information is based on intra-RNA cross-links (the arrowed lines marked with Roman numerals); RNA-protein cross-link sites (underlined protein numbers connected to the RNA by lines with arrowheads); RNA-protein foot-print sites (protein numbers without underlining, connected to the RNA by lines without arrow heads). A protein number in brackets indicates that the foot-print site was only at a single base. Suffixes a, b etc. distinguish different sites for the same protein. Foot-print sites for tRNA are indicated by filled triangle (A-site) and circles (P-site). The tRNA and mRNA cross-link sites are marked as tX or mX, respectively. [The figure is from Brimacombe (1995) with permission]

coli mRNAs possess a SD sequence (Shine and Dalgarno, 1974; Smiley *et al.*, 1982; Pirrotta, 1979; Ptashne *et al.*, 1976) located 5-8 bases upstream from the initiation codon AUG and it is essential for initiation of translation. The sequence 5'-AGGAGG-3', which is complementary to the 5'-CCUCCU-3' sequence present at the 3' end of *E. coli* 16S rRNA, is the sequence most frequently found in bacterial leader sequences (Gold *et al.*, 1981). The removal or displacement, even by a few bases, of a SD sequence can have a significant effect on the translational efficiency of mRNAs (Gold *et al.*, 1981).

As more and more transcription start sites are mapped, it is apparent that an increasing number of prokaryotic mRNAs have no functional SD signal due to very short or absent 5' untranslated leader regions (Wu and Janssen, 1996). Among these, many leaderless transcripts encode essential proteins that confer antibiotic resistance on bacterial cells (Bibb *et al.*, 1994). In addition, several natural and synthetic mRNAs have been shown to be translated in *E. coli* despite the absence of a SD interaction (Resch *et al.*, 1996; Wu and Jassen, 1996), which indicates the existence of alternative translation initiation mechanisms. Studies by RNA-RNA cross-linking experiments have also shown that other regions of 16S rRNA are involved in the interaction with mRNA during the initiation of translation step (McCarthy and Brimacombe, 1994). The complex interaction of mRNA with the *E. coli* ribosome is also illustrated by the existence of prokaryotic translational "enhancers". It has been shown in a series of papers that the 5' untranslated region of the tobacco mosaic virus RNA, which consists of a 68-base sequence (Omega), enhances translation *in vivo* and *in vitro* in both eukaryotes and prokaryotes (Gallie *et al.*, 1987; Gallie *et al.*, 1988; Gallie and Kado, 1989; Sleat *et al.*, 1987; Sleat *et al.*, 1988). Olins and Rangwala (1989) have found that a 10-base sequence (UUUAACUUUA, named "epsilon")

occurring naturally in the bacteriophage T7 gene 10 (*g10*) causes a 110-fold increase in the translation efficiency of the *lacZ* gene. The same sequence exists as well in other highly expressed genes of *E. coli* such as *atpE* (McCarthy *et al.*, 1985) and *recF* (Sandler and Clark, 1994).

Besides these alternative upstream signals, the "downstream box" may be of functional significance in several prokaryotic mRNAs. Enhancer sequences complementary to the rRNA have also been found to be downstream (nucleotides +15 to +26) of the initiation codon in the T7 gene *0.3* (Sprengart *et al.*, 1990) and T7 gene *1* (Helke *et al.*, 1993). The downstream box was originally described as a translational enhancer element of approximately 8-13 nucleotides that is complementary to 16S rRNA nucleotides 1469-1483 and located downstream of the initiation codon of various highly expressed *E. coli* and bacteriophage mRNA (Sprengart *et al.*, 1990). Studies related to the elucidation of the molecular effects of these "downstream box" enhancers enabled Sprengart *et al.* (1996) to formulate their hypothesis about the downstream box as an efficient and independent translation initiation signal in *E. coli*. Recent studies in our laboratory have also shown that the TMV omega sequence is not just a translational enhancer but is also a real initiator of translation interacting with the nucleotides 1343-1355 in 16S rRNA (Ivanov *et al.*, 1992; Ivanov *et al.*, 1995). Almost all alternative mRNA binding sites described so far are localized within an area between the head and body of the three-dimensional model of the 30S ribosomal subunit (McCarthy and Brimacombe, 1994). Thus, the conventional interaction between the SD sequence in mRNA and the 3' end of 16S rRNA may not be the only way to initiate translation in *E. coli*.

The interaction of mRNA to a region other than the SD of the 16S rRNA affects the

expression of a reporter gene. On the other hand, the expression level can also provide information on how mRNA and 16S rRNA molecules are interacting as well as how different regions of 16S rRNA interact with mRNA in a synergistic way (more than one binding site). In spite of much knowledge of detailed ribosomal structure (Fig.8), the final solution for the localization of the 530 loop and 1400 region of 16S rRNA in the 30S subunit is not available. Brimacombe and his colleagues (Dontsova *et al.*, 1992; Rinke-Appel *et al.*, 1993) reported that 530 loop and the 1400 region of the 16S molecule are close neighbours in the decoding area of the 30S subunit because the cross-links between mRNA and 16S rRNA were from position +4 of the mRNA to nucleotide 1402 of the 16S rRNA; from +7 to 1395 and from +11 to 532. However, Powers and Noller (1994) believe that the 530 loop and the 1400 region are widely separated. They argue that position 34 and 37 in the anticodon loop of tRNA have been cross-linked to nucleotide 1400 of the 16S rRNA and ribosomal protein S7, the 1400 region is thus considered to be close to S7. Further data from footprinting and neutron map experiments revealed that ribosomal protein S12, a reasonable marker for the position of the 530 loop, was 11.2 nm away from S7. The same group recently reported that the 530 loop and 1400 region are close using site-directed hydroxyl radical probing method but the explanation for the discrepancy by two different approaches was not available (Heilek and Noller, 1996).

Objectives

There are several biologically interesting reasons to study PAP: 1) the great potential of this gene product for use in human and veterinary medicine; 2) the control of plant virus diseases; 3) the use of PAP as an efficient inhibitor of prokaryotic and eukaryotic protein synthesis. The main objectives of this thesis are the following: 1) to isolate the PAP gene from pokeweed plants; 2) to express the PAP gene in *E. coli*; 3) to study the relationship between the structure of PAP and viral inhibiting activity and toxicity to *E. coli* cells; 4) to seek and characterize novel translational enhancers for overexpression of PAP; 5) to use the PAP gene for developing transgenic tobacco plants resistant to viruses and 6) to develop strategies for the use of PAP as an anti-HIV agent through gene therapy.

MATERIALS AND METHODS

Total Cellular RNA Extraction from Pokeweed Leaves

The original *Phytolacca americana* seeds and cuttings were kindly provided by L. Lamb, University of Waterloo, Waterloo, Ontario, Canada. Pokeweed leaves were harvested by Dr. Frank DiCosmo and were kept at -70^{0}C until use. Total cellular RNA was extracted from pokeweed leaves according to Ausubel *et al.* (1989). Twelve grams of leaves were frozen in liquid nitrogen and ground to powder in prechilled mortars. The powder was suspended in 10 ml of extraction buffer containing 0.2 M Tris-HCl pH 8.2, 0.1 M LiCl, 5 mM EDTA, 1% SDS. The slurry was vortexed and incubated in ice for 15 min and then extracted once with 5 ml TE buffer-saturated phenol, followed by adding 5 ml of chloroform and centrifuged at 9,100 *g* for 15 min. The aqueous phase was extracted with 10 ml chloroform and centrifuged as before. The upper phase was then extracted twice with an equal volume of chloroform and the RNA was precipitated with 2.5 volumes of 95% ethanol at -20^{0}C overnight. The precipitate was spun down at 9,100 *g*, 10 min in 50 ml corex tubes, the pellet was washed with 70% ethanol and air-dried for 15 min.

The RNA pellet was suspended in 400 µl of DEPC-treated H_2O and 400 µl of 4 M LiCl were added. The solution was incubated overnight at 4^{0}C and the precipitate was recovered after centrifugation at 8,160 *g* for 10 min at 4^{0}C. The precipitate was resuspended in 400 µl of DEPC-treated H_2O. Seventeen µl of 5 M NaCl (final concentration 0.2 M) and 3 volumes of cold 95% ethanol were added. The final RNA was recovered by centrifugation at 8,160 *g* for 10 min. After washing with cold 70% ethanol for 3 min, the RNA pellet was air-dried. The RNA pellet was

dissolved in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0) and analysed by electrophoresis in a 1% denaturing agarose gel containing 1X TBE buffer (0.1M Tris, 0.1M boric acid and 2 mM EDTA, pH 8.3) at 30 mA (the gels were pre-electrophoresed for 20 min before sample loading). Ribosomal RNA (16S and 23S rRNA) from *E. coli*, and tobacco mosaic virus (TMV) RNA were used as size markers. The RNA was visualized under an ultraviolet lamp after staining with 2 μg/ml ethidium bromide and the remaining RNA stored in TE buffer at -20°C.

Synthesis of Complementary DNA (Reverse Transcription)

Total cDNAs complementary to pokeweed poly-A mRNAs were synthesized according to a modified method of Gubler and Hoffman (1983). Synthesis was carried out in a 100 μl reaction volume containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM dithiothreitol (DTT), 8 mM dCTP, 2mM dTTP, 0.5 mM dATP, 50 μCi of [^{32}P]dATP (>3000 Ci/mmol; ICN), 60 Units RNasin (RNase inhibitor, Promega), 10 μg pokeweed RNA, 2.5 μg of Oligo(dT)$_{12\text{-}18}$ primer and 19 units of M-MLV reverse transcriptase derived from Moloney murine leukaemia viruses (Life Technologies). To anneal the primer to the template RNA, the pokeweed RNA and Oligo (dT)$_{12\text{-}18}$ primer mixture was boiled at 100 °C for 3 min and allowed to cool slowly to room temperature before the addition of the other reagents. The reaction mixture was then incubated at 40-42°C for 2 hrs, then supplemented with 1μl of 100 mM dATP and re-incubated at the above temperature for another 1.5 hrs. The reaction was terminated by addition of 20 mM EDTA, pH 8.0. The cDNA was precipitated with 2 M ammonium acetate and 2.5 volumes of 95% ethanol at -70°C. The cDNA was then phenol/chloroform (1:1 v/v) extracted and precipitated with 0.2 M potassium acetate and 2.5 volumes 95% ethanol. After precipitation, the cDNA was spun down and resuspended in 30 μl of double-distilled water. The cDNA sample was run on a gel and

autoradiographed.

Synthesis and Purification of Oligonucleotide Primers

Primers used for PCR amplification, nucleotide sequencing, and DNA duplex adaptors were designed and synthesized on a Cyclone™ DNA Synthesizer (MilliGen Biosearch). After the primers were synthesized, two 1 cc syringes were inserted at each end of the column containing the primer and 1 ml of NH_4OH (30%) was passed through the column at least 20 times to remove the primer from the sides of the column and into the solution. The column and syringes were incubated at 37°C for 1 hour. After passing the solution through the column several more times, the primers were removed from the column and injected into a 1.5 ml screw cap tube. The tube was wrapped with parafilm, incubated at 60°C overnight, and dried in a Savant Speed Vac SC110 for 3 hours. DNA was resuspended in 200 µl of distilled water, extracted with chloroform and then precipitated with 0.5 M NaCl and 2.5 volumes of 95% ethanol at -70°C for 10 min.

The primer was pelleted by centrifugation and resuspended in 100 µl double-distilled water. The oligomer solution was spotted 2.5 cm from the bottom of the glass coated with silica (10 x 10 x 0.25 cm, Merck) with a Pasteur pipette. Plates were loaded in the chromatography chamber containing a solution of 55% (v/v) n-propanol, 35% (v/v) ammonium hydroxide (30%) and 10% (v/v) distilled water. The solvent front was allowed to rise until it was approximately 1 cm from the top of the plate (about 3-4 hours). Plates were then removed from the chamber, laid out on paper towels and left to dry in a fumehood for 15 min. The primers were then visualized and their locations marked with a pencil by placing the plate under UV light. The silica within this region was then scraped off with a spatula and transferred to a 1.5 ml Eppendorf tube. Two hundred µl of distilled water were added and the tube vortexed and centrifuged at 12,000

g for 5 min at 4°C. The supernatant containing the DNA was collected in a second tube and the silica pellet was resuspended twice more in 100 µl H_2O, vortexed and centrifuged again, the supernatants were pooled together and then dried in a Savant Speed Vac SC110 to remove the NH_4OH and n-propanol. The white pellet was redissolved in 50 µl H_2O and centrifuged at room temperature for 3 min to pellet any remaining debris. The solution was extracted with chloroform and precipitated with 0.5 M NaCl and 2.5 volumes of 95% ethanol at -70°C for 5 min. DNA was pelleted by centrifugation in a microfuge, redissolved in 150 µl dH_2O and its concentration was determined using a spectrophotometer at a wavelength of 260 nm.

Amplification of cDNA Fragments by Polymerase Chain Reactions (PCR)

cDNA prepared above was used as templates for PCR. A 5' forward primer P_{PAPF} (5' GATGGATCCGATGAAGTCGATGCTTGTGGTGAC 3') and a 3' reverse primer P_{PAPR}(5' CGCGGATCCTGGTACCTTGCATCATAATTGGAAGTTTTATTTC 3') were designed based on published PAP cDNA sequence (Lin *et al.*, 1991). The 5' and 3' primers contained a unique *Bam*HI restriction site and both *Bam*HI and *Kpn*I restriction sites (underlined), respectively. The polymerase chain reaction was carried out in a 100 µl total volume using Vent^{exo+} (NEB) or Taq (Promega) DNA polymerase. The reaction cycle was repeated 35 times using a PTC-100™ Programmable Thermal Controller (MJ Research, Inc) under the following conditions depending on primers used: denaturation at 93°C for 30 seconds to 1 min, annealing from 50°C to 62°C for 30 seconds to 2 min and extension at 72°C for 2 min with $MgSO_4$ or $MgCl_2$ from 1.5 mM to 6 mM (final concentration). The PCR cDNA product was visualized by running on an agarose gel and confirmed by restriction endonuclease mapping.

Restriction Endonuclease Digestion of DNA

PCR products or plasmids containing cDNA inserts were digested with restriction endonucleases and the digestion conditions used were as recommended by the suppliers. About 0.2 μg of DNA was digested in a 20 μl reaction volume containing the appropriate buffer and enzyme (5-10 units). The mixture was incubated at an appropriate temperature for 1-3 hrs. Ten μl of digest were then mixed with 2 μl of 10x loading dye (0.125% bromophenol blue, 0.125% xylene cyanol FF, 50% glycerol, 50 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0), and loaded onto a 1% agarose gel containing 0.25 μg/ml ethidium bromide. Electrophoresis was performed in 1x TBE buffer at a constant current of 80 mA for about 1 hr. Lambda DNA digested with both *Hind*III and *Eco*RI was used as size markers.

Isolation of PAP DNA Fragments from Agarose Gels

One to three μg of plasmid DNA were digested with restriction endonucleases and subjected to electrophoresis on a 1% prestained agarose gel. The DNA fragment of interest was cut out with a razor blade from the gel in the form of an agarose block and placed in a 0.5 ml Eppendorf tube. Three holes had been pierced into the bottom of the Eppendorf tube with a needle, and siliconized glass wool had been packed into it prior to the addition of the agarose block. This tube was then placed inside a 1.5 ml Eppendorf tube, and the tubes centrifuged at 4000 *g* for 3 min. After centrifugation, 100 μl of TE buffer were added to the small tube, and the tubes were frozen first for 10 min at -20°C, and then for 10 min at -70°C. The tubes were left to thaw at room temperature for 15 min, and again subjected to centrifugation. The procedure was repeated three times more, and the resulting solution remaining in the large tube was extracted several times with a double volume of n-butanol until the final aqueous volume reached about

200 µl. The aqueous solution was extracted with an equal volume of phenol three times and then with an equal volume of phenol-chloroform (1:1) three times until the slurry between two phases was invisible. The top aqueous phase was then extracted with chloroform twice. The DNA recovered was precipitated in 0.5 M NaCl and 2.5 volumes of 95% ethanol, after incubation at -70°C for 5 min.

Cloning of cDNAs into Plasmid Vectors

(A) Single-digestion/dephosphorylation of plasmids

Two µg of plasmid were digested to completion with an appropriate restriction enzyme. The linear DNA was precipitated with 0.2 M NaCl and 3 volumes of 95% ethanol at -70°C, and resuspended in 40 µl 1x calf intestinal alkaline phosphatase (CIP) buffer (20 mM Tris, pH 8.0, 1 mM $MgCl_2$ and 1 mM $ZnCl_2$). One third of the DNA (0.33 µg) was dephosphorylated with addition of 0.1 or 1.0 U CIP and incubated at 50°C for 1 hr. The reaction was terminated by the addition of EDTA pH 8.0 to a final concentration of 5 mM and thereafter heated at 75°C for 10 min, then phenol/chloroform extracted and precipitated. The vector DNA was resuspended in 20 µl of TE^{-1} (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

(B) Synthetic duplex cassette inserts

Three µg of each oligonucleotide were mixed in 100 µl of sterile double-distilled water containing 10 mM $MgCl_2$. The mixture was boiled for 3 min in a water bath. The water bath was then allowed to cool to room temperature for 5 hr. The annealed duplex cassette containing sticky overhangs was precipitated by adding 5 M NaCl (final concentration of 0.2 M) and 3 volumes of 95% ethanol, and centrifuged at 16,000 *g* for 10 min. The pellet was rinsed with 70% ethanol

and air-dried. DNA concentrations were measured at OD 260 nm.

(C) Ligation of cDNAs into plasmid vectors

One hundred ng of vector DNA was mixed with 20 ng of PAP cDNA (4:1 molar ratio of insert DNA to vector DNA) in a 20 μl of reaction volume containing 1x ligation buffer and 2 units T4 DNA ligase. The ligation reaction was incubated at 16°C overnight. The ligated DNA was transformed into $CaCl_2$-treated competent *E. coli* cells.

Production of Competent Cells

Competent *E. coli* DH5α, LE392 and XL1-blue cells were prepared in the following manner: a single *E. coli* colony was added to a culture tube containing 2 ml of YT (16 g Bacto-tryptone, 10 g yeast extract and 10 g NaCl in 2 litres of distilled water, pH 7.0) and the tube was incubated overnight with shaking at 300 RPM, at 37°C. Three hundred μl of this culture were inoculated into a flask containing 30 ml of YT. The cells were incubated at 37°C until an optical density at 590 nm of 0.6 was reached (approximately 3 hours). The cells were pelleted by centrifugation at 1,500 *g* for 5 min, and gently resuspended in 15 ml (half the original volume) of 50 mM $CaCl_2$ at 4°C. The suspension was left on ice for 15 min and the cells were again pelleted by centrifugation. The cells were gently resuspended in 2 ml $CaCl_2$ (one fifteenth the original volume), and either maintained at 4°C until use, or aliquoted into a 100 μl volume (containing 15% glycerol) and stored at -70°C.

Competent *Agrobacterium tumefaciens* strain PMP90 cells were prepared in the same manner. Cells were inoculated in 3 ml of YT medium containing 30 μg/ml of streptomycin and

incubated at 30°C, shaking, for 4 days. One hundred µl of culture were inoculated into a flask containing 30 ml of YT. The cells were shaken for 8 hours at 28°C to reach an optical density at 590 nm of 0.6. The cells were pelleted and resuspended in 15 ml of 20 mM $CaCl_2$ at 4°C for 15 min. The cells were again pelleted and resuspended in 2 ml 20 mM $CaCl_2$ and maintained either at 4°C until use or at -70°C as aliquots (containing 15% glycerol).

Transformation of Competent Cells

(A) *E. coli* cells

Approximately 20-100 ng plasmid DNA and 100 µl of competent *E. coli* cells were added to a prechilled 1.5 ml Eppendorf tube and incubated on ice. After 15 min, the cells were heat-shocked in a 42°C water bath for 90 seconds. One ml of YT broth was added to the cells, and the tube was incubated at 37°C for 1 hour. Cells were pelleted by centrifugation in a microfuge, and all of the supernatant was discarded. Cells were resuspended in 100 µl of YT medium and plated out on 2 X YT medium with 1.5% agar. Depending on the requirements of specific plasmids and purposes, additional supplements were applied. For white/blue colony selection on the medium, 0.003% (w/v) X-gal (substrate of β-galactosidase), 0.04 mM isopropyl-β-D-thiogalactopyranoside (IPTG, inducer) and 100 µg/ml ampicillin were used. Cells which had been successfully transformed with the pBS^+ plasmid containing an insert appeared on the plate as white colonies, while blue colonies consisted of cells which contained the pBS^+ plasmid without the insert (Sambrook *et al.*, 1989). A number of different antibiotics were used in other cases.

(B) *Agrobacterium tumefaciens* PMP90 cells

Competent *Agrobacterium tumefaciens* strain PMP90 cells were transformed according to

the procedure of Holster *et al.* (1978). One μg of plasmid DNA was added to a 0.1 ml aliquot of competent *Agrobacterium tumefaciens* cells in an Eppendorf tube. The mixture was frozen with liquid nitrogen and heat-shocked at 37°C for 5 min. One ml of YT medium was added to the tube and incubated at 28°C for 4 hours. The cells were pelleted in the microfuge and resuspended in 0.1 ml of YT medium. The cells were spread on an agar plate containing 25 μg/ml streptomycin, 5 μg/ml of tetracycline and 30 μg/ml kanamycin, and incubated at 28°C for 3-4 days.

Alkaline Extraction of Plasmid DNA

(A) Small scale isolation (Miniprep)

Colonies were inoculated into 2 ml YT broth containing the appropriate antibiotic (100μg/ml ampicillin, 30 μg/ml streptomycin, 25 μg/ml kanamycin and 5-10 μg/ml tetracycline) and incubated at 37°C overnight with shaking. Plasmid DNA was extracted from these cells using the procedure of Sambrook *et al.* (1989). Cells were pelleted by centrifugation at 12,000 *g* for 20 seconds in a microfuge and pellets frozen at -70°C for 10 min. The cells were thawed, and resuspended in 150 μl Solution I (50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl, pH 8.0 plus freshly added 3 mg/ml lysozyme) on ice. After 15 min, 300 μl Solution II (0.2 N NaOH plus fresh 1% SDS) were added and the tubes were inverted gently and left on ice for another 15 min, then 225 μl Solution III (3 M sodium acetate, pH 4.8) were added and the tubes were left on ice for a further 15 min. All larger materials which had precipitated out of solution were then pelleted by centrifugation in a microfuge at 4°C for 12 min at 12,000 *g*. Plasmid DNA was precipitated out by incubating the supernatant in an equal volume of isopropanol for 5 min at -70°C, then centrifuged at 12,000 *g* for 10 min and the pellet was redissolved in 100 μl TE buffer. One hundred μl of 5 M ammonium acetate were added and the mixture was incubated on

ice for 20 min to overnight. The large macromolecules were pelleted and discarded after centrifugation at 8,160 *g*. Plasmid DNA in the supernatant was precipitated by adding 2 volumes of 95% ethanol. The pellet was finally extracted with phenol/chloroform and precipitated again.

(B) Large-scale isolation (Maxiprep)

Thirty ml of YT containing appropriate amounts of antibiotics were inoculated with 20 µl of overnight culture and shaken at 37°C overnight. Cells were harvested at 9,100 *g* (10K RPM, Sorvall SS34 rotor) for 5 min and resuspended in 3 ml Solution I containing 3 mg/ml fresh lysozyme. After the cell suspension was incubated on ice for 15 min, two volumes Solution II (6 ml) were added to the suspension and incubated on ice for another 15 min. One and a half volumes Solution III (4.5 ml) were then added to the extract, mixed by swirling gently and kept on ice for 15 min. Denatured chromosomal DNA and protein were pelleted by centrifugation at 9,100 *g* for 20 min. The supernatant was decanted into another fresh tube. One volume of ice-cold isopropanol was added to the supernatant and the mixture was incubated at -70°C for 10 min. The DNA precipitate was recovered by centrifugation at 9,100 *g* for 10 min. After rinsing with 70% ethanol, the DNA pellet was air-dried and dissolved in 50 µl of TE. The DNA samples were further purified by ammonium acetate and phenol/chloroform as described above for miniprep.

Long Term Storage of Cells in Glycerol Stocks

Both transformed and non-transformed strains of *E. coli* and *Agrobacterium tumefaciens* PMP90 cells were grown in 2 ml overnight cultures in YT broth containing the appropriate

antibiotics at 37°C and 28°C, respectively, with shaking. The following day, cells were pelleted by centrifugation at 12,000 *g* in a microfuge for 1 min, and resuspended in a well-mixed solution of 0.5 ml (50%) 10 mM magnesium sulfate and 0.5 ml (50%) sterile glycerol. This cell suspension was then stored in a -70°C freezer.

Sequencing Double-stranded DNA Templates

Dideoxyribonucleotide DNA sequencing protocol was according to the instructions provided by US Biochemicals (sequenase kit, version 2.0). About 1 μg of plasmid DNA was denatured in 0.2N NaOH/0.2 mM EDTA (final concentration) at 37°C for 30 min and neutralized with 0.1 volume of 3 M sodium acetate, pH 4.5. Denatured DNA was then precipitated by the addition of 3 volumes of 95% ethanol, stored at -70°C for 5 min and centrifuged for 10 min in a microfuge. The denatured DNA was rinsed with ice-cold 70% ethanol, air-dried and resuspended in 7 μl distilled water. Seven μl (1 μg) of this DNA were then mixed with 2 μl 5x sequenase reaction buffer (5x: 200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) and 1 μl (50-100 ng) of primer. The mixture was then boiled for 3 min and rapidly cooled in ice water. One μl of 0.1 M DTT, 1 μl of manganese chloride, 1 μl ^{35}S-dATP (10 mCi/ml, Amersham) and 2 μl diluted (1:5) dGTP labelling mix (5x =7.5 μM each of dGTP, dCTP, dTTP) were added to the DNA mixture. The mixture was then placed at room temperature and 2 μl diluted (1:8) sequenase (1x enzyme dilution buffer: 10 mM Tris-HCl pH 7.5, 5 mM DTT, 0.5 mg/ml BSA) were added. After 5 min, four 3.8 μl aliquots were transferred and each immediately mixed with a different dideoxynucleotide termination solution (80 μM each of all four dNTPs, 50 mM NaCl and 8 μM of the particular dideoxynucleotide) at 37°C. After incubation for 5 min at 37°C, a 4 μl aliquot of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol

blue, 0.05% xylene cyanol FF) was added. Before loading, each reaction tube was boiled for 3 min, rapidly chilled in ice water for 5 min, centrifuged for 30 seconds. Three µl aliquots were loaded onto an 8% sequencing gel [42 g urea, 20 ml 40% acrylamide/bis (19:1), 20 ml 5x TBE, 0.5 ml 10% ammonium persulphate, 20 µl TEMED and distilled water to 100 ml]. Sequencing gels were poured at least 2 hours prior to use and were pre-electrophoresed in 1x TBE for 30 min at a constant power of 75 Watts. Wells were rinsed free of urea. Electrophoresis was run on a BRL sequencing apparatus (model S2) at a constant power of 65 Watts until the bromophenol blue reached the bottom of the gel. The gel was then carefully placed into 10% ethanol/10% glacial acetic acid (v/v) fixative and left for 1 hr. Drying was performed at 80^{0}C under a vacuum for 2 hrs and the gel placed in contact with X-ray film (Kodak XAR5 or Amersham MP) for 2-5 days.

Construction of vectors for constitutive and inducible expression of the PAP gene

Both constitutive (pP_tR_9) and inducible ($pP_tO_tR_9$) vectors are based on the cloning plasmid pBR322 in which either a strong synthetic promoter (P_t) and a consensus Shine-Dalgarno (SD) sequence (R_9), or a promoter (P_t), synthetic *lac* operator (O_t) and SD sequence (R_9) were substituted for the original *Eco*RI/*Hind*III fragment (Rommens *et al.*, 1983; Ivanov *et al.*, 1987a and 1987b). To bring a *Bam*HI site next to the SD sequence so that the full-length PAP gene (flanked by *Bam*HI sites) can be inserted at the *Bam*HI site, the *Xho*I/*Bam*HI fragment (a part of the Tc^r gene) was removed and a *Bam*HI site was introduced by substituting a synthetic *Xho*I/*Bam*HI fragment (carrying the same SD sequence)(Fig.9). Thus, two complementary oligonucleotides, P_{SDF} (5'TCGACAA-GGAGGTTTG3') and P_{SDR} (5'GATCCAAACCTCCTTG3')

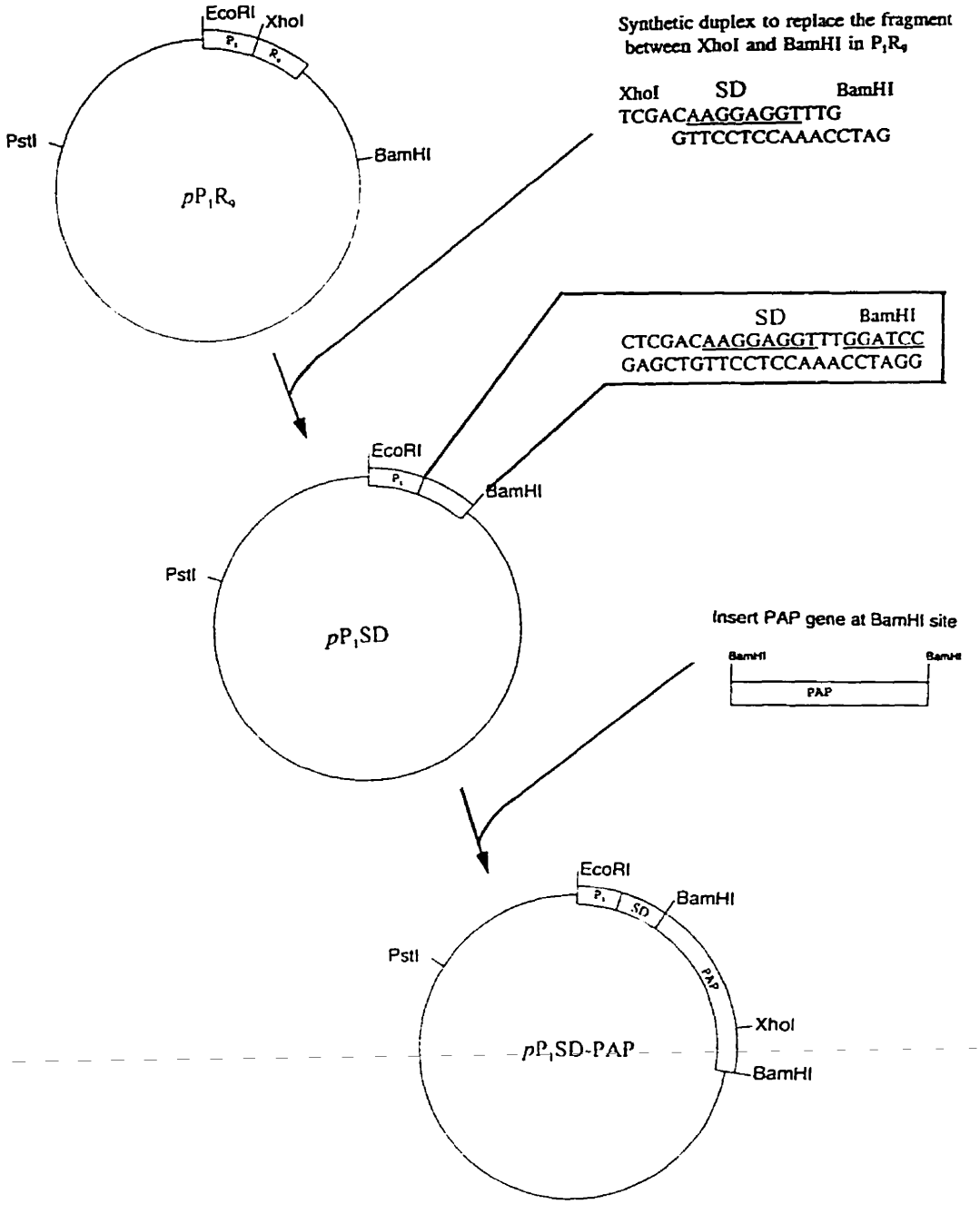


Fig. 9. Construction of the full-length PAP gene in a vector derived from pP_1R_9. A pBR322 derived expression vector (pP_1R_9) was digested with *Xho*I and *Bam*HI, and the resulting fragment released was replaced with a synthetic duplex containing a consensus Shine-Dalgarno sequence (SD, 5'AAGGAGGT3'). The modified plasmid was referred to as pP_1SD. A cDNA fragment encoding the entire PAP gene was released from clone pBS^+-PAP_{anti} by *Bam*HI digestion, and was inserted into the *Bam*HI site of pP_1SD. Orientation of the full-length PAP gene was determined by both restriction enzyme analysis and nucleotide sequencing analysis. pP_1SD-PAP was one of the PAP expression vectors. For detail sequence see Fig.17.

were synthesized. Twenty ng of the annealed cassette, and 100 ng of pP_tR_9 vector DNA digested with *Xho*I and *Bam*HI and eluted from a gel by centrifugation (50:1 molar ratio of the cassette and the vector DNA), were used in a 40 μl ligation reaction volume. Plasmids containing the insert were screened by digestion with *Xho*I and *Sal*I. After modification of the plasmid pP_tR_9, the full-length PAP gene (*PAP*$_f$) was inserted at the *Bam*HI site. Sense and antisense orientations of the insert were determined by restriction enzyme digestions. A similar procedure was applied for the inducible plasmid $pP_tO_tR_9$.

Mutations to the PAP Gene

(A) Deletion of the N-terminal signal peptide (22 amino acids)

The expression vector containing the entire PAP gene including its N-terminal 22 amino acids was chosen for further mutational study. To delete the N-terminal signal, a 5' primer $P_{\Delta N}$ (5'GATGGATCCGATGGTGAATACAATCATCTACAAT3') containing a *Bam*HI site (underlined) and a new start codon ATG (underlined) was synthesized. PCR was carried out using the above 5' primer and the 3' primer P_{PAPR} (5'CGCGGATCCTGGTACCTTGCATCAT AATTGGAAGTTTTATTTC3') used for cloning of the full-length PAP gene from single stranded cDNA. The reaction was under the following conditions: denaturation at 93°C for 1 min, annealing at 52°C for 1 min and extension with Vent polymerase at 72°C for 2 min. The PCR fragment, after digestion with *Bam*HI, was ligated into the linear pP_tSD cut with *Bam*HI. Orientation of the truncated PAP gene was determined by the size of fragments released after digestion with *Pst*I and *Xho*I.

(B) Deletion of the C-terminal regions (62 amino acids)

Two primers, $P_{\Delta CF}$ (5' TCGAGCTATGATGA**AGATCT**T 3') and $P_{\Delta CR}$ (5' CTAGAAG ATCTTCATCATAGC 3'), were synthesized, purified and annealed. The annealed double-stranded cassette contained *Xho*I and *Xba*I overhangs, and introduced a unique *Bgl*II restriction site (AGATCT in bold) for screening, as well as two consecutive stop codons TGATGA (underlined) which resulted in a deletion of 62 amino acids (including 29 amino acids of the C-terminal extra peptide) (Fig.23).

Ten μg of vector pP_1SD-PAP was digested overnight in a 400 μl volume containing 100 units of *Xho*I and *Xba*I (located in the C-terminal end of PAP gene but unique in the entire vector). The digested vector DNA was eluted from a 1% agarose gel. Twenty ng of vector DNA was ligated overnight with 5 ng of synthetic oligonucleotide cassette DNA. The ligation mixture was transformed into *E. coli* LE392 cells. Colonies containing C-terminal truncated PAP gene were determined by digestion with *Pst*I and *Bgl*II (releasing a 1.7 kb fragment).

Complementarity Search between 5' untranslated mRNA and 16S rRNA of *E. coli*

The complementarity between 5' non-coding region of mRNAs and 16S rRNA of *E. coli* was analyzed using the software program SEQAID (Kansas State University, Molecular Genetics Laboratory, version 3.81).

Mutations of Boxes (in 5' untranslated mRNA) Complementary to 16S rRNA of *E. coli*

(A) A polylinker of plasmid pP_1-PL

Mutations in box I, box II or both were created by PCR in two steps (Fig.26). The first

step was to mutate the pP_1-PL vector without the PAP gene and the second was to insert the PAP gene at the *Bgl*II site of those mutated plasmids. To this end, a 5' primer P_{PstI} (5'GTAAACTGC AGGCATCGTGGTGTCA 3') was designed to contain *Pst*I site (underlined) and complementary to the *Pst*I site region of pP_1-PL. Three 3' primers, containing mutated box I (PM_{boxI}, 5' TCTAG AA**GATCT**-TTAATA-AGTAGGTTCAAATTTA 3'), box II (PM_{boxII}, 5'TAGA**AGATCT**AACT CGA-TATTATA-CAAATTTATGAA3'), and both boxes ($PM_{boxI\&II}$, 5' TCTAGA-**AGATCT**-TTAATAATATTATA-CAAATTTATGAATCTATTATACAG3'), respectively, as well as a *Bgl*II restriction site (in bold), were designed to be complementary to the *Bgl*II site region of the plasmid pP_1-PL. PCR was carried out using combinations of the 5' primer and one of the three 3' primers, pP_1-PL as a template and Taq DNA polymerase. The PCR reaction was repeated for 1 min at 93°C, 1 min at 56°C and 2 min at 72°C for 35 cycles. A sticky PCR fragment (after digestion with *Pst*I and *Bgl*II) was used to replace the original (wild-type) fragment between *Pst*I and *Bgl*II in plasmid pP_1-PL. After transformation, colonies with mutations were screened by digestion with *Xho*I and *Pst*I (the *Xho*I site was destroyed due to the mutations). The clones which were not digested by *Xho*I were selected.

The PAP cDNA fragment released from clone pBS^--PAP_{anti} by *Bam*HI digestion was then inserted into the *Bgl*II site of the mutated plasmids as described above. The presence and the orientation of the insert were determined by digestion with *Pst*I and *Xho*I.

(B) A 33-nucleotides fragment from potato virus X (PVX)

Two boxes complementary to 16S rRNA were also mutated similarly to the method described above (Fig.30). Two primers were designed; the 5' end primer PM_{PVX} (5' CTAGTCT

AGATTAAACGGTTATATATAATATAATACTCGAAAGatgaag 3') was complementary to *Xba*I site region except for the two mutated boxes (underlined) of plasmid P_1-PVX-PAP. The 3' end primer P_{SphI} (5' AGGTTGAGGCCGTTGAGCA 3') was complementary to a sequence located downstream of the PAP gene in the same plasmid. PCR was performed using both above primers and Taq polymerase. The reaction was repeated at 93°C for 1 min, 56°C for 1 min and 72°C for 2 min for 35 cycles. The sticky PAP cDNA fragment containing the mutated boxes after digestion with *Xba*I and *Hind*III was ligated between *Xba*I and *Hind*III sites of P_1-PL and the resulting clone was designated P1-PVXm-PAP.

Determination of mRNA yield and mRNA stability

E. coli LE 392 cells transformed with corresponding expression plasmids were cultured in YT supplemented with 50 μg/ml ampicillin and 0.2% glucose at 37°C until a cell density of OD_{590nm} = 2.0 (about 8 h of incubation). Bacterial cells were harvested from 10 ml of cultures and total mRNA was isolated according to Current Protocols (Ausabel *et al.*, 1989).

RNA was treated with DNase (RNase-free), deproteinized and precipitated with ethanol. The RNA was dissolved in distilled water (pre-treated with DEPC) and the concentration of RNA was determined by an OD_{260nm} measurement. Forty μg RNA evaporated to dryness in a Speed Vac centrifuge were dissolved in 20 μl of a denaturing solution (300 mM NaCl + 30 mM sodium citrate + 10% formaldehyde, pH 7.0). After heating at 60°C for 15 min, samples were spotted onto nitrocellulose filters. The filters were baked, prehybridized and hybridized according to Ivanov and Gigova (1987). Hybridization was carried out using a γ^{32}P-labeled oligonucleotide, P_{Hybrid}, 5'-GATATTGTCACCACAAGC-3' which is complementary to the 5' PAP gene coding

region. After hybridization, filters were cut and the ^{32}P-radioactivity was measured by Cerenkov counting in a Beckman scintillation counter. Three independent experiments were repeated.

To study the *in vivo* stability of mRNA, RNA synthesis was blocked by rifampicin and nalidixic acid (Ivanov *et al.*, 1992). 20 ml LB medium containing 50 μg/ml ampicillin were inoculated with an overnight culture (1:50 by vol.) and cultivated with vigorous shaking at 37^{0}C for 1 hour. Nalidixic acid and rifampicin were added to final concentrations of 0.5 μg/ml and 60 μg/ml, respectively. Bacterial cells were harvested at the following time intervals: 0, 10, 20, 30, 40, 50, 60, 70, 80, 120, 180, 240, 300, 500, 600 seconds. Samples were frozen at -70^{0}C, thawed on ice and spun down at 4^{0}C. Cells were resuspended in ice-cold solution containing 50 mM sodium acetate, pH 5.0, 1 mM EDTA and 0.5% SDS. Total cellualr RNA was isolated, spotted onto nitrocellulose filters and hybridized with ^{32}P-labelled oligonucleotides specific for the corresponding mRNA. The radioactivity of each spot was measured and plotted versus the time. The half-life of mRNA was defined as the time corresponding to a 50% decrease in radio-activity of each spot compared with the level of the zero-time control.

Analysis of Expressed PAP

(A) PAP preparations from *E. coli* and transgenic tobacco plants

E. coli LE392 cells harbouring both sense and antisense orientations of the PAP gene were cultured overnight at 37 ^{0}C in TB or YT medium. Cells were pelleted by centrifugation at 12,000 *g* for 5 min, sonicated and pelleted again. Ammonium sulphate was added to the supernatant to reach a final saturation of 35% and the mixture stirred for 15 min at room temperature. Precipitate was removed by centrifugation at 12,000 *g* for 15 min and the procedure repeated,

with an increase to 85% saturation of ammonium sulphate. The pellet was resuspended in double distilled water, dialysed and lyophilised. Total protein concentration was measured by OD_{280} nm using bovine serum albumin (BSA) as a standard.

Tobacco leaf tissue (0.3 g) was ground using liquid nitrogen. One ml of protein extraction buffer [0.3 M Tris-HCl, pH 8.3, 15 mM DTT (dithiothreitol), 1 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 mM Benzidine] was added to the tissue powder and the supernatant was collected by centrifugation at 11,750 *g* for 15 min. Trichloroacetic acid was then added and the mixture was incubated on ice for 1 hour. After centrifugation at 2,000 *g* for 5 min, the protein pellet was resuspended in 500 µl acetone. The suspension was centrifuged again at the same speed and the pellet was vacuum-dried, resuspended in 0.1 M sodium carbonate and 0.1 M DTT and incubated overnight at 4°C. The sample was finally denatured with sample buffer which will be described below. In general, 0.1 g of leaf tissue produced 50 µg total leaf protein.

(B) Preparation of anti-PAP antibody

Pure PAP from Calbiochem-Novabiochem Corporation was used to inoculate a male New Zealand rabbit (2.5 Kg). The rabbit was injected intramuscularly with 1 mg of PAP protein in a final volume of 1 ml (half volume of protein solution + half volume of incomplete Freund's adjuvant). The rabbit's immune response was boosted every 4 weeks for three times as above. The rabbit was sacrificed by cardiac puncture. The blood was allowed to clot for two hours at 37°C. The blood clot was separated from the sides of the centrifuge tubes, and placed at 4°C overnight to shrink the clot. The tubes were centrifuged and the serum collected. Sodium azide (0.01%) was added to the antiserum for storage at -20°C in 1.5 ml Eppendorf tubes. The specificity and titre of the antibody was examined by Western dot blot. One µg of pure PAP from

pokeweed plants, 1 μg of BSA and 10 μg of BSA were spotted on a strip of nitrocellulose membrane. Membranes were incubated with anti-PAP antiserum at 1:1000, 1:3000 or 1:5000 dilutions (see below for detailed immunoblot protocol). The most diluted titre which still produced positive signal was used for other immunoblot analyses.

(C) Western blot

Protein lysates were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a mini slab gel apparatus according to the method of Laemmli (1971). The 12-15% separating gels and 5% stacking gels were prepared according to the manufacturer (Bio-Rad). The gels were allowed to polymerize for about two hours. Equal volumes of sample solution and 2x sample buffer (10% β-mercaptoethanol, 60 mM Tris-HCl, 1 mM EDTA, 2% SDS, 12% sucrose, pH 8.3) were mixed and boiled for 10 min. The denatured protein were loaded in each lane.

The running buffer was 50 mM Tris, 0.38 M glycine, 0.1% SDS, pH 8.3. A current of 20 mA per plate was applied for electrophoresis. The proteins were separated on slab gels until the leading dye-front reached or completely passed the bottom of the separating gel.

Western blots were performed according to the instructions from Bio-Rad. After proteins were separated on an SDS-PAGE gel, the gel was soaked in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for half an hour and then placed in a Trans-Blot cell (Bio-Rad) at 30 volts overnight. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was washed with Tris-buffered saline (1x TBS: 20 mM Tris, 500 mM NaCl, pH 7.5) for 15 min and then blocked with 2% gelatin in TBS for half an hour, followed by incubation with anti-PAP rabbit antiserum at 1:3000 dilutions for 2 hr. The membrane was

washed in Tween 20, Tris-buffered saline (1x TTBS: 20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5) for 15 min with three changes, and then incubated with a secondary antibody, goat anti-rabbit antibody conjugated with alkaline phosphatase (Life Technologies) at 1:3000 dilution for 1 hour. The membrane was washed again with TTBS for 15 min with 2 changes and once with TBS. The membrane was finally soaked in a substrate buffer containing 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$ (TNM) and developed in 20 ml of substrate buffer TNM containing 88 µl NBT (nitroblue tetrazolium chloride) and 66 µl BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt).

(D) Immuno dot blot

PAP concentrations derived from *E. coli* were estimated from a series of 4-fold successive dilutions on a nitrocellulose membrane. Three hundred µg of total protein was dissolved in 160 µl TBS. Forty µl were transferred to the next well containing 120 µl TBS and the same dilution process was repeated. Known quantities of purified PAP from pokeweed plants containing equal amounts of lysate proteins of *E. coli* cells without the PAP gene (pP_I-SD) were used as a standard. The concentration of PAP was calculated as compared to a PAP standard.

Biological Activity Assays

(A) *In vitro* translation inhibition assay

In vitro translation experiments were carried out with wheat germ extracts (Promega). Wheat germ extracts were preincubated for 20 min at room temperature with lysates from *E. coli* LE 392 cells which expressed PAP. A lysate which did not express PAP was included as a negative control. Concentrations of 100 pg and 10 pg of PAP were used. Four µg of brome

mosaic virus (BMV) RNA templates were added to the wheat germ extract in the presence of L-[^{35}S]-methionine (1099 Ci/mmole, ICN) in a total volume of 50 µl and incubated for 60 min at room temperature. Translation products were subjected to electrophoresis on 12.5% polyacrylamide gels and exposed to X-ray film for 24 hours.

(B) Local lesion assay

Partially purified *E. coli* lysates containing PAP (clone pB96-47) were added to a suspension of tobacco mosaic virus (final virus concentration 50 µg/ml in 0.1 M phosphate buffer pH 7.2) to obtain final PAP concentrations of 0.0625 µg/ml, 0.125 µg/ml, 0.25 µg/ml, 0.5 µg/ml, 1.0 µg/ml, 2 µg/ml and 4 µg/ml. To eliminate the effect of *E. coli* lysates on local lesion production, similar amounts of *E. coli* protein in the presence or absence of PAP were added. A volume of 200 µl of *E. coli* lysate and TMV mixture was then rubbed on tobacco plants, (*Nicotiana glutinosa)* a local lesion host for the virus. A total of 12 randomly chosen half-leaves were used for each treatment. Local lesions were counted and the percent of inhibition of lesion formation was calculated using the following equation:

$$\% \text{ inhibition} = \left(1 - \frac{\text{No. of Lesions (PAP+TMV)}}{\text{No. of Lesions (TMV alone)}} \right) \times 100$$

Ultrastructural Immuno-gold Labeling

Immunogold labelling of *E. coli* was carried out as follows: *E. coli* LE392 cells were grown overnight in TB medium (one litre containing 12 g bacto-tryptone, 24 g yeast extract, 4 ml of glycerol, 2.3 g KH_2PO_4, 12.5 g K_2HPO_4), pelleted by centrifugation, and resuspended in

2 mL TB, 2 ml fixative (4% glutaraldehyde + 2% paraformaldehyde in a 100 mM phosphate buffer, pH 7.5). Cells were incubated for 3 hours at room temperature, pelleted again by centrifugation and resuspended in 4 mL fixative. Cells were embedded in LR White resin (Polysciences, Inc) and thin-sectioned onto 300 mesh copper grids using an ultramicrotome. Sections were indirectly immunolabeled by floating the grids on a succession of five 25 μl drops containing blocking buffer [1% BSA, 20 mM Tris HCl, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 0.2% gelatin] at room temperature for 1 hour, then with PAP antiserum added at a 1:30 dilution for 4 hours and finally in a buffer containing colloidal gold conjugated anti-rabbit antibody (Cedarlane) at a 1:100 dilution for 30 min. Ten nanometre gold particles were used in this study. Five minute washes in double distilled water were included at each step. Controls performed included 1) incubation with colloidal gold conjugated anti-rabbit antibody alone; 2) incubation with preimmune serum instead of anti-PAP antiserum and 3) the use of *E. coli* LE 392 cells which did not express PAP. Sections were stained in saturated uranium acetate for 8 min, rinsed in distilled water, then stained in 3% Reynolds lead citrate for 8 min. Samples were observed with a Philips 300 transmission electron microscope.

Construction of Recombinant Plasmid of pGA643 with Full-length and C-terminal Deleted Forms of the PAP Gene for the Development of Transgenic Plants

(A) Full-length PAP gene

The construction of the full-length PAP gene into binary vector pGA643 was described in Fig.10. The plasmid pBS^+-PAP_{anti} was first cleaved by unique *Bam*HI and *Sac*I to divide the PAP gene into two fragments (233 bp and 922 bp) with both sticky *Bam*HI and *Sac*I ends. Both

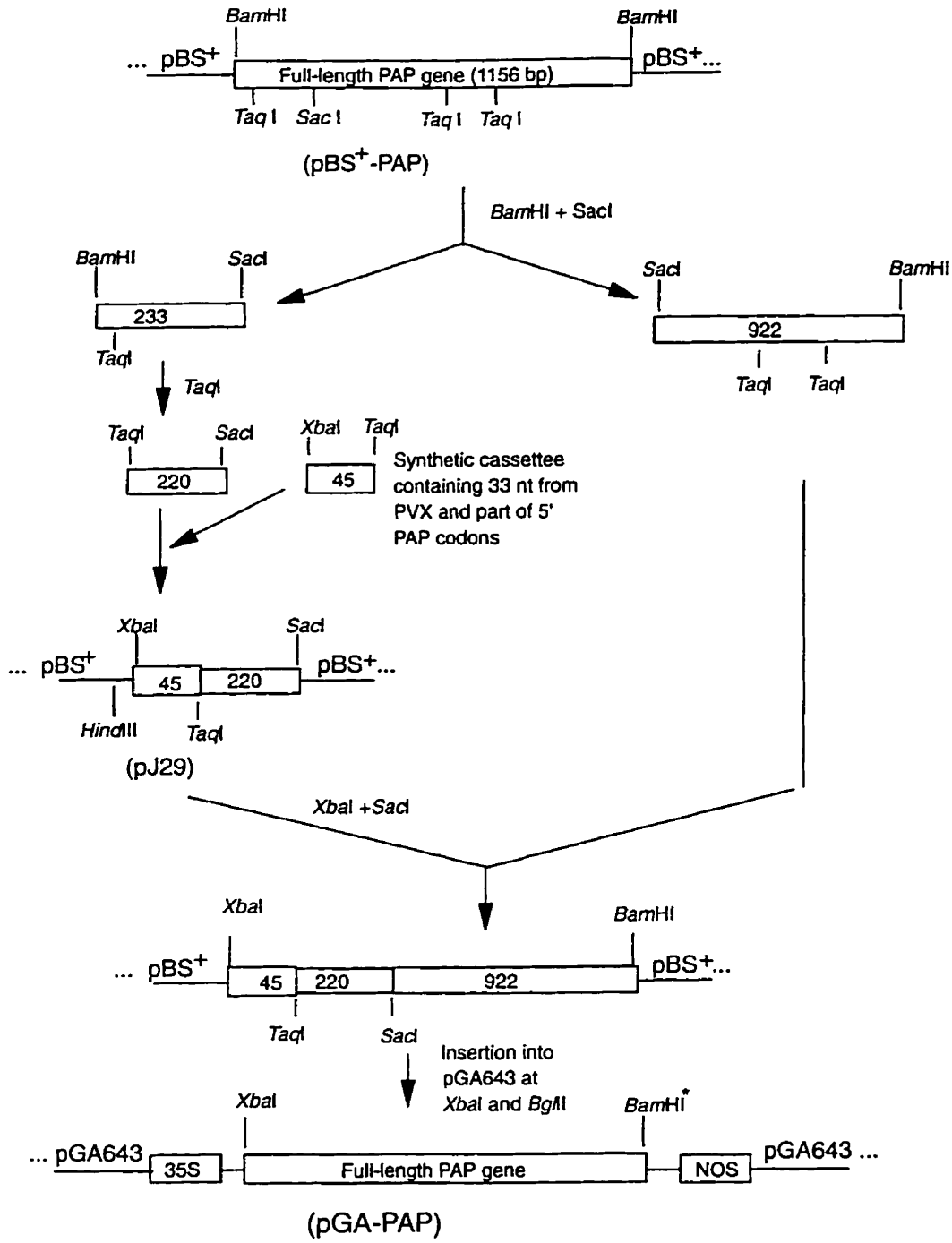


Fig.10. Construction of the full-length PAP gene into binary vector pGA643. For efficient expression of the PAP gene, a synthetic ribosome binding site (RBS) from PVX was integrated with the PAP gene released from the plasmid pBS$^+$-PAP$_{anti}$ after digestion with *Bam*HI. The recombinant PAP gene with the RBS from PVX was inserted at *Xba*I and *Bgl*II sites of the binary vector pGA643. 35S, a strong constitutive promoter from cauliflower mosaic virus; NOS, Nopaline synthase gene terminator. * *Bam*HI and *Bgl*II are compatible.

generated fragments were eluted from a 1% agarose gel. The smaller fragment (233 bp) was further digested with *Taq*I (*Taq*I is unique in the 233 bp fragment) to produce a smaller segment (220 bp) with sticky *Taq*I and *Sac*I overhangs. Two oligonucleotides, P_{RBSF} (5'CTAGATTGA-ACGGTTAAGTTTCCATTGATACTCGAAA**GATGAAGT**3'), and P_{RBSR} (5'CGACTTATCTTT-CGAGTATCAATGGAAACTTAACCGTTCAAT3'), were annealed to form a DNA duplex cassette. This cassette contained (1) thirty-three nucleotides (a ribosome binding site) derived from upstream of the coat protein gene of PVX, (2) eight nucleotides (in bold) from the start codon region of the PAP gene, and (3) a sticky *Taq*I overhang (lost after digestion with *Taq*I) and a sticky *Xba*I overhang (underlined). Both the duplex cassette and the 220 bp fragment were ligated into pBS^+ at *Xba*I and *Sac*I sites to form a 265 bp segment containing the ribosome binding site (RBS) and the sequence encoding the N-terminus of the PAP protein. After confirmation by sequencing, both the 265 bp fragment released after digestion with *Xba*I and *Sac*I, and the 922 bp fragment prepared previously were ligated into pGA643 at *Xba*I and *Bgl*II sites (*Bam*HI and *Bgl*II are compatible), but the *Bgl*II site was destroyed.

(B) C-terminal deleted forms of the PAP gene

C-terminal deleted forms of the PAP gene were isolated from an *E. coli* expression vector (Fig.11). These expression vectors include pC36, pC131 (from Alice Meng) and $pPAP_{\Delta C}62$ (the numbers represent the amino acids removed from the C-terminus). Three μg of each plasmid were digested with *Sal*I or *Sac*I. The resulting truncated PAP gene fragments were eluted from an agarose gel. One μg of pBS^+ was digested with *Hind*III and *Sal*I to produce a linear vector with sticky *Hind*III and *Sal*I overhangs. Two μg of pJ-29 containing PVX RBS (Fig.10) and sequence

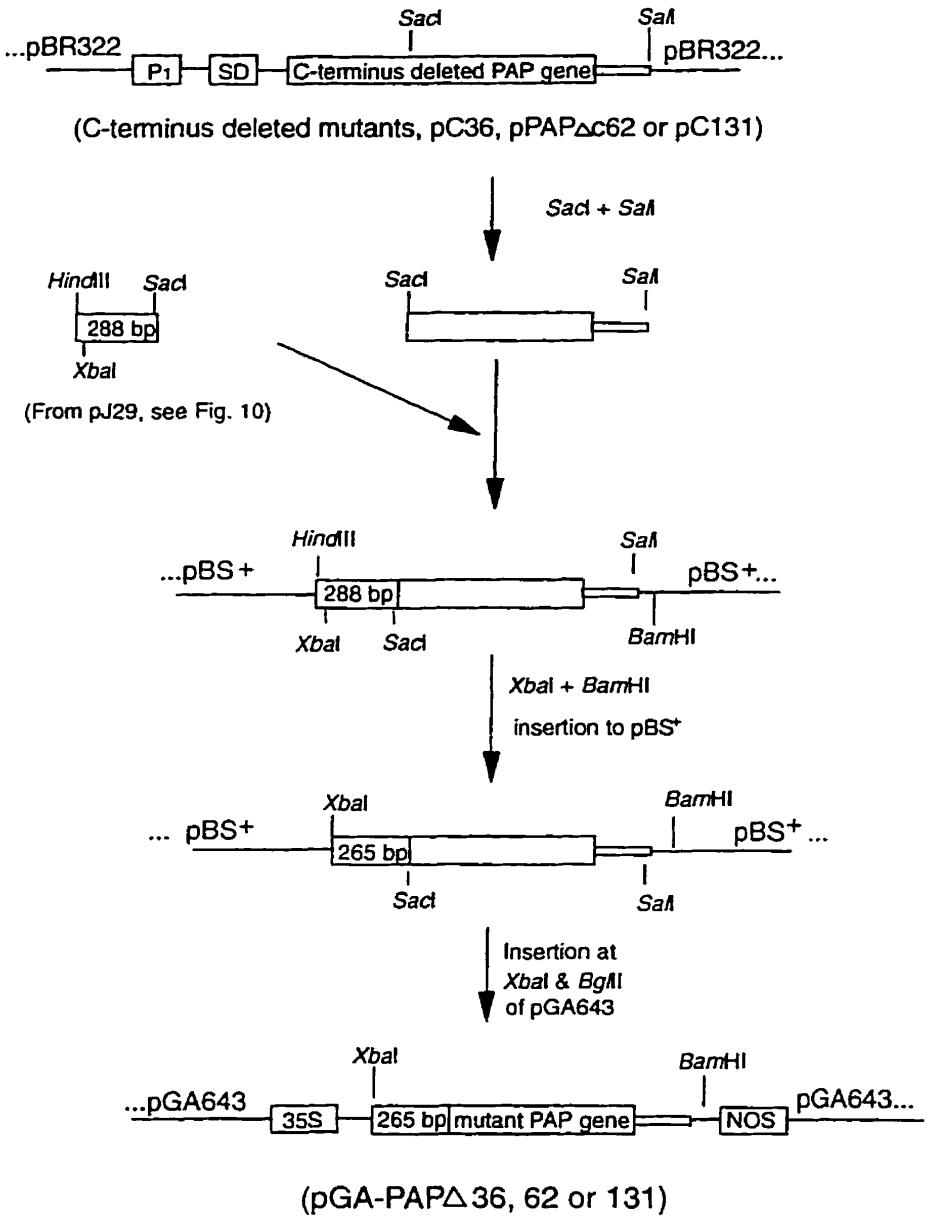


Fig.11. Construction of C-terminal deleted forms of the PAP gene into pGA643. PAP mutants, pC36, $pPAP_{\Delta C}62$ and pC131 were *E. coli* expression vectors and contained serial deletions of 36, 62 131 codons from the C-terminus of the PAP gene, respectively. These truncated forms of the PAP gene and the ribosome binding site from PVX were inserted at *Xba*I and *Bgl*II sites similar to Fig.10.

encoding the N-terminus of the PAP gene were digested with *Hind*III + *Sac*I and a 288 bp fragment was generated. The 288 bp fragment and one of the truncated PAP gene fragments were ligated into pBS$^+$ at *Hind*III and *Sal*I sites. The recombined fragment was cleaved after digestion by *Bam*HI (present in the pBS$^+$) and *Hind*III, and ligated into *Hind*III and *Bgl*II sites of pGA643 in a manner similar to the full-length PAP construction.

Transformation and Regeneration of Tobacco Plants

Both full-length and C-terminal deleted forms of the PAP genes were transformed into *Agrobacterium tumefaciens* strain PMP90. Sterile tobacco *Nicotiana tabacum* cv. Xanthi (a local lesion host for TMV) leaves were dissected into large squares (5 cm^2) and the edges and petioles were trimmed. The leaves derived from the greenhouse were first sterilized by dipping in 70% ethanol and soaking in 1% bleach for 20 min. The leaves were rinsed 3 times with sterilized distilled water and the leaf edge was trimmed. The trimmed leaves were then applied on the surface of MS41 (Murashige and Skoog medium (MS41) containing 4.4 g/L salts, 3% sucrose, 1 mg/L 6-benzyl-aminopurine (BAP), 0.4 mg/L naphthaleneacetic acid (NAA) and 9g/L agar pH 5.8 for 2 days under the condition of 24^{0}C, constant light. The leaf pieces were cut into smaller ones (2 x1 cm) and the old edges were trimmed again. *Agrobacterium tumefaciens* strain PMP90 cells transformed with plasmids pGA (pGA643 without the PAP gene used as a control), pGA-PAP (pGA643 with the full-length PAP gene), pGA-PAPΔ36 (pGA643 containing the PAP gene with a deletion of 36 codons from the C-terminus), pGA-PAPΔ62 and pGA-PAPΔ131 (similar to pGA-PAPΔ36 except 62 and 131 codons instead of 36 codons, respectively) were grown at 28^{0}C overnight in 30 ml of YT medium containing 30 μg/ml streptomycin, 30 μg/ml kanamycin and 10 μg/ml tetracyline. The cells were pelleted after centrifugation and resuspended in fresh

60 ml (1:2 dilution) YT medium. The leaf pieces were then incubated with 30 ml of bacterial suspension for 5 min. The pieces were blot-dried with filter paper and transferred to fresh plates containing the same medium (MS41). After 3 days or 6 days co-culture under the same condition as above, the leave pieces were transferred onto selection medium MS41a (MS41 + 100 mg/L kanamycin and 400 mg/L carbenicillin). Primary shoots that developed after 3-4 weeks were cut off and transferred to a rooting medium MS 42 (2.2 g/L MS salts, 1.5% sucrose, 0.9% agar, pH 5.8, 100 mg/L kanamycin and 400 mg/L carbenicillin). Rooted plants were transferred to soil and kept under polyethylene bags and high humidity for about 2 weeks before transfer to soil in the greenhouse. The antiviral activity of the transgenic plants was performed as follows. Stock TMV (20 mg/ml) was diluted with 0.1 M phosphate buffer, pH 7.2, to a final concentration of 3 μg/ml. Two hundred μl of diluted TMV were inoculated onto the potential transgenic plants. Inoculation was carried out on ten plants of each line and three leaves derived from each plant. The local lesion number was counted and calculated.

External Toxicity Assay of PAP with or without Retroviruses on Mammalian Cells

The external toxicity assay of PAP on mammalian cells was performed by Mansoora Ahmed in Dr. Joshi's laboratory). Briefly, PA317 cells were harvested and resuspended in αMEM medium (Eagle's minimal essential medium) supplemented with 10% fetal bovine serum, antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) and 2 mM glutamine. *E. coli*-expressed PAP was added into the 3 ml of cell suspension (containing 3 x 10^5 cells) to bring final PAP concentrations to 0, 0.05, 0.5 or 5 μg/ml. The contents were transferred to a 60 mm plate and grown in a 5% CO_2 environment at 37°C. The confluences of cell colonies were recorded on the third day.

The same PA317 cells were tested when both purified PAP and retroviral vector particles released from Psi-2 cell lines were applied. PA317 cells were seeded in 60 mm plates so that they were 50% confluent before infection. One hundred μl of retroviral vector particles, an appropriate amount of PAP to bring final PAP concentrations to 0, 0.05, 0.5 or 5.0 μg/ml, were mixed with the appropriate volumes of non-selective medium (a total volume of 400 μl from PAP and non-selective medium) and 500 μl of non-selective medium containing 16 μg/ml polybrene to bring the final volume to 1 ml. After incubating for 2 hr, 4 ml fresh non-selective medium was added and the cells continued to grow. On the second day, the infected cells were washed with phosphate buffered saline (PBS), trypsinized, transferred to 100 mm plates and grown in selective medium containing 200 μg/ml G418. The selective medium was changed every 3-4 days and the remaining resistant cells were counted on the 15th day.

Transfection of Retroviral Vectors into Mammalian Cells

(A) Construction of N-terminal signal deleted PAP gene ($PAP_{\Delta N}$) into retroviral vectors

Two primers were designed and made according to the known cDNA sequence of the PAP gene. The upstream primer, $P_{HIV\Delta NF}$ (5'CCCCCGGGCCAAGTCGGCCGCCACCATGGT-GAATACAATCATCTACAAT3') starts from the mature protein sequence with the addition of an *Sfi*I recognition sequence, consensus Kozak ribosome-binding-site, GCCGCCACC (Kozak, 1989) and an ATG start codon (underlined). The downstream primer, $P_{HIV\Delta NR}$ (5'TTCCCCCGGGCCAAGTCGGCCTTATGATCAGAATCCTTCAAATAGA3') starts 5 bases after the stop codon of the opposite strand with an additional *Sfi*I recognition sequence (underlined). The above two primers were used to amplify the PAP gene without its leader sequence. Each PCR product was verified by digestion with restriction enzymes *Acc*I, *Bst*XI or

*Sac*I distributed in different places of the PAP gene. The amplified PCR fragment was digested with *Sfi*I and ligated into the *Sfi*I sites of MoTN-Ti-Tat-Ri (TR) retroviral vector to yield a PAP recombinant plasmid which is designated as A_1.

As a control, a compatible synthetic DNA duplex cassette was prepared. Two oligonucleotides, $P_{HIV\Delta PAPF}$ (5'ATCGATCCGGATCCTCGCGAGCGGCCGCACT3'), and $P_{HIV\Delta PAPR}$ (5'GCGGCCGCTCGCG-AGGATCCGGATCGATACT3'), were synthesized and the cassette containing unique restriction sites, *Cla*I, *Bam*HI, *Nru*I, *Not*I, *Acc*III (*Bsp*EI) and compatible *Sfi*I overhangs on both ends was prepared and cloned into *Sfi*I site of the retroviral vectors as described above. One of the PAP gene deleted retroviral vectors, designated as A_2, was used as a control.

Preparation of plasmids for transfection was mainly performed according to the procedure of Maniatis *et al.* (1989). *E. coli* strain LE392 containing the desired vectors grown in 3 ml of YT medium supplemented with 100 μg/ml ampicillin overnight. The overnight culture was then added into 500 ml of fresh YT medium containing 100 μg/ml ampicillin and continued to grow for another 16 hrs. The cells were harvested by centrifugation at 2000 *g* for 10 min and resuspended in 4 ml of Solution I (50 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 15% sucrose). One ml of freshly-made 10 μg/ml lysozyme in 0.25 M Tris-HCl pH 8.0 was added to the suspension and the mixture was incubated in ice for 15 min. Five ml of a detergent solution (0.1% Triton X-100, 50 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0) were added and mixed by gentle inversion until the solution became viscous. The resulting solution was then centrifuged at 15,000 *g* at 4^0C for 40 min and the supernatant was collected. CsCl was dissolved in the supernatant to reach a final concentration of 1 g/ml. The solution was transferred to Beckman Quick-Seal centrifuge tubes with the use of a 5 ml syringe and 18G needle, and 0.4 ml of 10

mg/ml ethidium bromide solution was added to each tube. Tubes were then sealed and ultracentrifuged at 55,000 RPM at 16°C for 60 hrs (Beckman L8-55 Ultracentrifuge, 70.1 Ti rotor). A major band containing plasmid DNA near the bottom of the centrifuge tube was collected with a 5 ml syringe and 18G needle and extracted with isobutanol (1:1) until the ethidium bromide was removed (approximately four times). TE solution (pH 8.0) was added to the extract to a volume of 5 ml and 2.5 volumes of 95% ethanol were added. The DNA solution was incubated at -20°C overnight and precipitated at 15,000 *g* at 4°C for 20 min. After washing with 70% ethanol, the DNA pellet was resuspended in sterile TE solution (pH 8.0).

(B) Transfection of Psi-2 packaging cells

Transfection assays were carried out essentially as described by manufacturer's instructions (CellPhect Transfection Kit, Pharmacia). Ecotropic Psi-2 packaging cells (3×10^5) were seeded onto 60 mm plates so that they were 50% confluent prior to transfection. The old medium was removed and replaced with 3 ml fresh medium. The DNA precipitates were prepared as follows: retroviral plasmid DNA (1 μg) was diluted to a final volume of 120 μl with sterile distilled water. An equal volume of Buffer A [0.5 M $CaCl_2$, 0.1 M N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid), HEPES)] was added and the mixture vortexed and incubated at room temperature for 10 min. Then, 240 μl of Buffer B (0.28 M NaCl, 0.05 M HEPES, 0.75 mM NaH_2PO_4, 0.75 mM Na_2HPO_4) was added, the mixture vortexed briefly and then incubated at room temperature for 15 min. The DNA precipitates were added dropwise onto the cells without removing the medium and the cells were incubated for 6 hrs at 37°C in a humidified atmosphere with 5% CO_2. The cells were washed twice with fresh medium, glycerol-shocked with 1.5 ml 15% glycerol in 10 mM HEPES pH 7.5, 150 mM NaCl for 3 min at room temperature, then

washed once with fresh medium. Five ml of fresh medium were added and the cells were grown at normal conditions for 2 days. On the third day, the transfected cells were washed once with phosphate-buffered saline (PBS) containing antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B), trypsinized with 0.05% trypsin, 0.53 mM EDTA, transferred to 100 mm plates and grown in selective medium (αMEM supplemented with 10% fetal bovine serum (Hyclone), antibiotic-antimycotic solution as above with 2 mM glutamine and 200 μg/ml G418 antibiotic. The medium was changed every 2-3 days. Fourteen days after transfection, the resistant cells were washed with PBS, trypsinized and re-seeded at a 1/10 dilution while the remainder was frozen in 90% fetal bovine serum and 10% dimethyl sulfoxide (DMSO).

Transfected Psi-2 cells were grown in selective medium on 100 mm plates until 50% confluent. Five ml of fresh medium without G418 replaced the old selective one. Cells continued to grow overnight and the medium which contained retroviral vector particles released from the transfected packaging cells were collected with a syringe and stored. After sterilation with a filter (0.22 μm, Millipore), the vector particles were stored at -70^{0}C.

Infection of PA317 Packaging and CD4$^+$ Cells with Vector Particles

Since vector particles from the Psi-2 packaging cell line only infect murine (rat and mouse) cells, in order to produce vector particles with wide host range including human cells, amphotropic PA317 packaging cells (3 x 10^5) were seeded in 60 mm plates and grown until 50% confluence before infection. Vector particles were thawed at room temperature and placed on ice. One hundred μl of vector particles were mixed with 400 μl of non-selective medium, and 500 μl non-selective medium containing 16 μg/ml polybrene (Sigma). The cells were grown for 2 hr

first, then 4 ml of fresh non-selective αMEM was added. The cells were grown overnight at 37 ^{0}C. The following day, the infected cells were washed with PBS, trypsinized and transferred to 100 mm plates and grown in selective medium (200 μg/ml G418). The medium was changed every 3-4 days until the 14th day. Vector particles were collected the same as for Psi-2 cell line.

Three x 10^{5} MT4 cells were pelleted and resuspended in 0.5 ml RPMI 1640 medium containing 16 μg/ml polybrene. One hundred μl of vector particles were added to the cells. Non-selective RPMI 1640 medium was then added to bring total volume to 1 ml. The cells were transferred to 60 mm plates and incubated at normal growth conditions for 2 hr. Four ml of fresh non-selective medium were added and the cells continued to grow. On the second day, the cells were pelleted and resuspended in a selective medium containing 400 ug/ml G418 and transferred to 100 mm plates. Selective medium was changed to get rid of dead cells. By day 14, all of the uninfected cells had died and the remaining resistant cells were stored in liquid nitrogen in 90% fetal bovine serum and 10% DMSO.

Total Genomic DNA and RNA Isolation from Mammalian Cells

About 10^{7} MT4 cells were harvested by centrifugation at 88 *g* for 3 min at room temperature in the microfuge. The pelleted cells were washed with ice-cold phosphate buffered saline (PBS) twice and then resuspended in 1 ml of extraction buffer containing 10 mM Tris, pH 8.0, 0.1 mM EDTA pH 8.0, 0.5% SDS, 100 μg/ml proteinase K and 20 μg/ml RNase A. After incubating at 37^{0}C overnight, the suspended cells were transferred to a 15 ml centrifuge tube and TE buffer (pH 8) was added to a final volume of 3 ml. Three ml of phenol, pH 8.0 were added and vortexed. Denatured materials such as proteins were pelleted by centrifugation at 4,000 RPM for 5 min. The phenol extraction step was repeated once more. The aqueous phase containing

genomic DNA was transferred to another 15 ml tube and extracted with a 3 ml mixture of phenol/chloroform (1:1) once and chloroform (chloroform:isoamyl alcohol = 24:1) 3 times. The DNA was precipitated by adding an equal volume of isopropanol and 300 µl 2 M sodium acetate pH 5.2. After freezing at -70°C for 10 min, the DNA was pelleted by centrifugation at 9,100 *g* for 20 min and washed with cold 70% ethanol twice and dried. The DNA was finally dissolved in 200 µl of TE pH 8.0 and the concentration was measured at OD_{260}.

Total cellular RNA was extracted from mammalian MT4 cells for the detection of PAP mRNA. Approximately 2 x 10^7 cells were harvested by centrifugation at 88 *g* for 5 min. The cell pellet was washed with 5 ml of PBS twice and resuspended in 350 µl solution D (4 M Guanidine thiocyanate, 25 mM sodium citrate and 0.5% Sarcosyl, heated to 65°C) and 5 µl β-mercaptoethanol. After vortexing, a second solution containing 350 µl phenol, 350 µl chloroform (chloroform:isoamyl alcohol = 24:1) and 35 µl 2 M sodium acetate was added and vortexed for 1 min. The aqueous phase was collected after centrifugation at 8,160 *g* for 10 min in the microfuge and 350 µl of ice cold isopropanol was added. The mixture was kept at -70°C for 15 min and the RNA was pelleted after centrifugation at 8,160 *g* for 10 min. The pelleted RNA was then resuspended in 350 µl solution D, 35 µl 2 M sodium acetate, 350 µl isopropanol and 5 µl β-mercaptoethanol. After incubation at -70°C for 15 min, the RNA was repelleted after centrifugation at the same speed and the pellet was washed with 75% ethanol and vacuum-dried for 15 min. RNA concentration was measured by spectrophotometry based on OD_{260}. For long storage, the RNA was resuspended in 25 µl of 0.5% SDS and RNases were inactivated by heating at 65°C for 10 min.

HIV-1 Infection

One hundred or five hundred μl of live HIV-1_{NL4-3} preparation was used to infect 10^6 MT4 cells transformed with PAP construct or devoid of PAP gene in 1 ml of RPMI 1640 medium containing L-glutamine, antibiotics, 10% fetal calf serum and 400 μg of G418. After incubating at 37°C for 2 hr, cells were washed to remove unabsorbed virus particles and allowed to resume growth. The selective medium was changed every 3-4 days until the 15th day. The remaining resistant cells were tested for p24 antigen (a unique HIV-1 protein) by enzyme-linked immunosorbent assay (ELISA).

***In vitro* Transcription and Translation of $PAP_{\Delta N}$ Gene Cassette from Retroviral Vectors**

(A) Subcloning of Kozak-$PAP_{\Delta N}$ cassette into pBS^+

The N-terminal deleted PAP gene with Kozak ribosome binding site sequence was released from two types of retroviral vectors A_1 and α_1 [α1 is the same as A1 except lack of Ri (Rev responsive sequence) and the non-functional PAP gene (deletion of G_{21}) in the retroviral vector] after digestion with *Asp*718, and *Asp*718 + *Pst*I, respectively. The released PAP fragments were then ligated into their compatible restriction sites (*Asp*718, *Asp*718 + *Pst*I respectively) of plasmid pBS^+ so that the PAP gene was introduced downstream of the T7 promoter. White colonies were collected and analyzed by restriction analysis of *Xho*I + *Pst*I, *Asp*718 + *Sac*I (for subclones derived from A_1) and *Asp*718 + *Pst*I (for subclones derived from α_1). One subclone from each type of retroviral vectors was designated as pSA_1 and $pS\alpha_1$. Large-scale preparations of these two clones were made, purified with ammonium acetate and extracted with phenol/chloroform. After linearization with *Xba*I (to produce full-length PAP mRNA) and *Bst*XI

(to produce truncated and inactive PAP mRNA), the plasmids were treated once more with phenol/chloroform.

(B) *In vitro* transcription

Transcription reactions were performed as described by Beck *et al.* (1990). A 100 µl reaction volume was prepared containing 25 ng/µl of linearized template DNA pSA_1 and $pS\alpha_1$, 1x transcription buffer (40 mM Tris-HCl, pH 7.5, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine), 0.5 unit/µl RNA guard (Pharmacia), 0.1 µg/µl RNase-free acetylated BSA (Promega), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.05 mM GTP, 0.5 mM m^7GpppG cap (NEB) and 1 unit/µl T7 RNA polymerase (NEB). After incubation for 15 min at 37°C, GTP was added to a final concentration of 0.3 mM and incubation continued at the same temperature for another 1 hr. Ten µl of RNA transcripts were analysed by electrophoresis on a 1% agarose gel pre-run for 30 min at 30 mA constant current and run for 30 min at the same power. After observing the transcripts, 2 µl of RQ-1 RNase-free DNase was added to the rest of the reaction and incubated for another 10 min at the same condition. The RNA transcripts were finally extracted with phenol/chloroform and the concentrations were measured at OD_{260nm}.

(C) *In vitro* translation

In vitro translation was performed according to the instructions provided by kit suppler (Promega) with some modifications. Rabbit reticulocyte lysates were preincubated with *in vitro* transcribed full-length or truncated mRNAs for 20 min at 30°C in the presence of L-[^{35}S]-methionine (1099 Ci/mmole). One reaction, in which no transcribed mRNA was added, was used as a positive control. After 20 min, 0.5 µl of CAT mRNA templates provided by the kit was added to each reaction and continued to incubate for another 40 min at 30°C. Translation products were then subjected to autoradiography.

RESULTS

I Isolation and Expression of the Full-length PAP Gene from Pokeweed Leaves

(A) Isolation of the full-length PAP gene

Total cellular RNA (1.5 mg) was isolated and purified from pokeweed plant leaves and the quality of the RNA preparation was examined by agarose gel electrophoresis (Fig.12, lanes 3 and 4). Five μg of RNA was then reverse-transcribed producing single-stranded cDNAs using

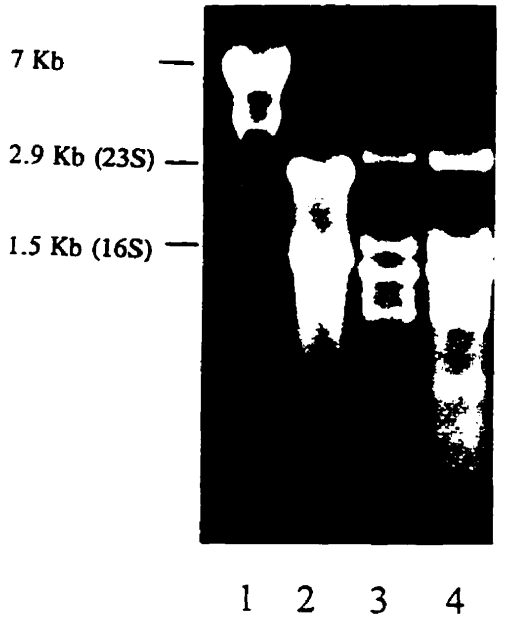


Fig.12. Electrophoretic profile of total cellular RNA from pokeweed leaves. Ten μg of TMV RNA (lane 1), 20 μg of rRNA from *E. coli* (lane 2) and total cellular RNA from pokeweed leaves (lane 3, 10 μg; lane 4, 20 μg) were run on a 1% denaturing agarose gel and stained with ethidium bromide. Note: Top two ribosomal RNA bands (lane 3 and lane 4) indicating good quality of the RNA preparation.

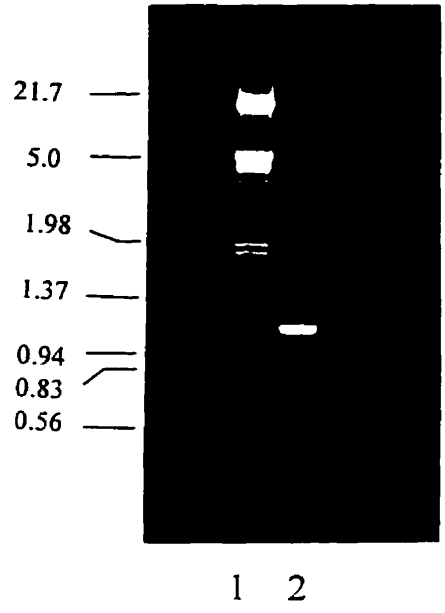


Fig.13. Polymerase chain reaction (PCR) product. PCR was performed under the following conditions: denaturation at 93°C for 1 min, annealing at 62°C for 2 min and extension at 72°C for 2 min. The reaction was repeated for 35 cycles. Ten out of 100 µl of PCR product were loaded onto a 1% agarose gel stained with ethidium bromide. Lane 1: DNA size marker in kb (λ DNA digested with *Hind*III and *Eco*RI); Lane 2: PCR product.

oligo-nucleotide $d(T)_{12-18}$ as a primer. The presence of the reverse-transcribed cDNA (α^{32}P[dATP] labelled) was confirmed by autoradiography. The above synthesized single-stranded cDNA was used as a template for polymerase chain reactions (PCR). Two primers were synthesized based on the published sequence (Lin *et al.*, 1991). Of the different annealing temperatures (55°C to 66°C) tested, 62°C was found to be optimal and produced a sharp, strong and homogenous band (Fig.13). Annealing temperatures that were too low (55°C) or too high (66°C) generated either

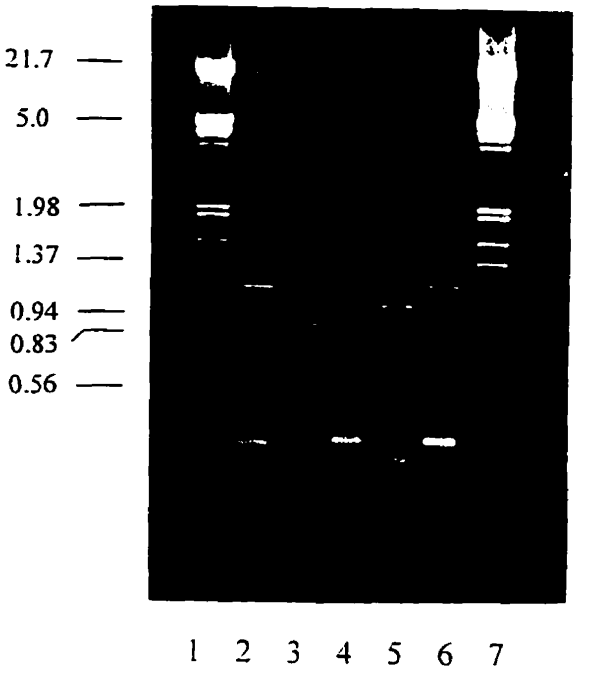


Fig.14. Restriction analysis of the putative PAP PCR product. The PCR product was digested with *Acc*I (lane 3), *Eco*RI (lane 4), *Hae*III (lane 5) and *Sac*I (lane 6) respectively. Lanes 1 and 7: DNA size markers in kb (λ DNA digested with *Hind*III and *Eco*RI). Lane 2: PCR product without endonuclease digestion.

smearing or no product as determined by gel electrophoresis. The size of the major PCR product was about 1.1 kb which was very close to that expected from the published sequence. To verify whether the PCR product contained the full-length PAP gene, it was analyzed by restriction endonuclease mapping before cloning into the vector pBS$^+$. Digestion with restriction endonuclease *Acc*I, *Eco*RI, *Hae*III and *Sac*I released fragments of expected sizes (in bp), i.e., 923 for *Acc*I, 626+522 for *Eco*RI, 1040 for *Hae*III and 958 for *Sac*I (Fig.14). Digestion with these enzymes also demonstrated that the minor band in the PCR product came from the 3' end of the PAP gene. After digestion with *Bam*HI, the PCR product was ligated into linearized pBS$^+$ that

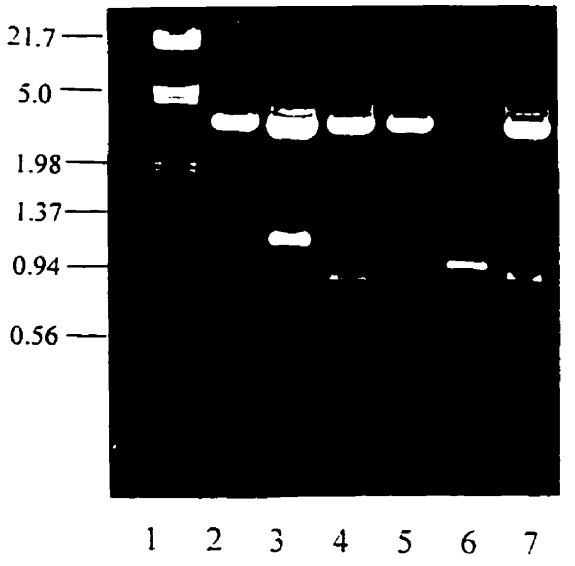


Fig.15. Restriction endonuclease analysis of recombinant plasmid pBS$^+$-PAP$_{anti}$. PAP cDNA in clone pBS$^+$-PAP$_{anti}$ (full-length PAP gene in the antisense orientation) was digested with different restriction enzymes. Lane 1, DNA size markers (Kb); lane 2, pBS$^+$ digested with *Bam*HI (as a control); lanes 3-7, pBS$^+$-PAP$_{anti}$ digested with different enzymes; lane 3, *Bam*HI; lane 4, *Bam*HI and *Acc*I; lane 5, *Bam*HI and *Eco*RI; lane 6, *Bam*HI and *Hae*III; and lane 7, *Bam*HI and *Sac*I.

had been cleaved by *Bam*HI. Analysis of DNA obtained from seven clones by *Bam*HI digestion showed that six clones contained a fragment of the correct size (1.1 kb). Further restriction mapping demonstrated that all six clones showed identical banding patterns to that of the PCR product prior to cloning (Fig.15). The cloned insert (PAP cDNA) from one of the six clones (pBS$^+$-PAP$_{anti}$) was subsequently sequenced using six different primers (Ppapseq1,2,3,4,5,6 listed in *Appendices*) and the resulting sequence obtained (Fig.16) was identical to that published (Lin *et al.*, 1991).

```
          10          20          30          40          50          60
          •           •           •           *           *           •
GGATCCG ATG AAG TCG ATG CTT GTG GTG ACA ATA TCA ATA TGG CTC ATT CTT GCA CCA ACT
BamHI   Met Lys Ser Met Leu Val Val Thr Ile Ser Ile Trp Leu Ile Leu Ala Pro Thr

          70          80          90         100         110         120
          •           •           •           *           •           •
TCA ACT TGG GCT GTG AAT ACA ATC ATC TAC AAT GTT GGA AGT ACC ACC ATT AGC AAA TAC
Ser Thr Trp Ala Val Asn Thr Ile Ile Tyr Asn Val Gly Ser Thr Thr Ile Ser Lys Tyr

         130         140         150         160         170         180
          *           *           •           •           *           *
GCC ACT TTT CTG AAT GAT CTT CGT AAT GAA GCG AAA GAT CCA AGT TTA AAA TGC TAT GGA
Ala Thr Phe Leu Asn Asp Leu Arg Asn Glu Ala Lys Asp Pro Ser Leu Lys Cys Tyr Gly

         190         200         210         220         230         240
          *           •           •           *           *           •
ATA CCA ATG CTG CCC AAT ACA AAT ACA AAT CCA AAG TAC GTG TTG GTT GAG CTC CAA GGT
Ile Pro Met Leu Pro Asn Thr Asn Thr Asn Pro Lys Tyr Val Leu Val Glu Leu Gln Gly

         250         260         270         280         290         300
          *           *           •           *           *           •
TCA AAT AAA AAA ACC ATC ACA CTA ATG CTG AGA CGA AAC AAT TTG TAT GTG ATG GGT TAT
Ser Asn Lys Lys Thr Ile Thr Leu Met Leu Arg Arg Asn Asn Leu Tyr Val Met Gly Tyr

         310         320         330         340         350         360
          *           •           *           *           *           •
TCT GAT CCC TTT GAA ACC AAT AAA TGT CGT TAC CAT ATC TTT AAT GAT ATC TCA GGT ACT
Ser Asp Pro Phe Glu Thr Asn Lys Cys Arg Tyr His Ile Phe Asn Asp Ile Ser Gly Thr

         370         380         390         400         410         420
          *           *           •           *           *           •
GAA CGC CAA GAT GTA GAG ACT ACT CTT TGC CCA AAT GCC AAT TCT CGT GTT AGT AAA AAC
Glu Arg Gln Asp Val Glu Thr Thr Leu Cys Pro Asn Ala Asn Ser Arg Val Ser Lys Asn

         430         440         450         460         470         480
          •           *           *           *           *           •
ATA AAC TTT GAT AGT CGA TAT CCA ACA TTG GAA TCA AAA GCG GGA GTA AAA TCA AGA AGT
Ile Asn Phe Asp Ser Arg Tyr Pro Thr Leu Glu Ser Lys Ala Gly Val Lys Ser Arg Ser

         490         500         510         520         530         540
          •           *           •           *           •           •
CAA GTC CAA CTG GGA ATT CAA ATA CTC GAC AGT AAT ATT GGA AAG ATT TCT GGA GTG ATG
Gln Val Gln Leu Gly Ile Gln Ile Leu Asp Ser Asn Ile Gly Lys Ile Ser Gly Val Met
```

Fig.16. Sequence of pokeweed antiviral protein cDNA in plasmid pBS$^+$-PAP$_{anti}$. PAP cDNA was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR). The PCR product was cloned into the *Bam*HI site of plasmid pBS$^+$.

(Continued on next page)

```
          550           560           570           580           590           600
           *             •             •             *             *             *
TCA TTC ACT GAG AAA ACC GAA GCC GAA TTC CTA TTG GTA GCC ATA CAA ATG GTA TCA GAG
Ser Phe Thr Glu Lys Thr Glu Ala Glu Phe Leu Leu Val Ala Ile Gln Met Val Ser Glu

          610           620           630           640           650           660
           *             *             *             *             •             •
GCA GCA AGA TTC AAG TAC ATA GAG AAT CAG GTG AAA ACT AAT TTT AAC AGA GCA TTC AAC
Ala Ala Arg Phe Lys Tyr Ile Glu Asn Gln Val Lys Thr Asn Phe Asn Arg Ala Phe Asn

          670           680           690           700           710           720
           *             *             *             *             *             *
CCT AAT CCC AAA GTA CTT AAT TTG CAA GAG ACA TGG GGT AAG ATT TCA ACA GCA ATT CAT
Pro Asn Pro Lys Val Leu Asn Leu Gln Glu Thr Trp Gly Lys Ile Ser Thr Ala Ile His

          730           740           750           760           770           780
           *             *             •             •             *             •
GAT GCC AAG AAT GGA GTT TTA CCC AAA CCT CTC GAG CTA GTG GAT GCC AGT GGT GCC AAG
Asp Ala Lys Asn Gly Val Leu Pro Lys Pro Leu Glu Leu Val Asp Ala Ser Gly Ala Lys

          790           800           810           820           830           840
           *             *             •             •             •             *
TGG ATA GTG TTG AGA GTG GAT GAA ATC AAG CCT GAT GTA GCA CTC TTA AAC TAC GTT GGT
Trp Ile Val Leu Arg Val Asp Glu Ile Lys Pro Asp Val Ala Leu Leu Asn Tyr Val Gly

          850           860           870           880           890           900
           •             •             *             *             *             •
GGG AGC TGT CAG ACA ACT TAT AAC CAA AAT GCC ATG TTT CCT CAA CTT ATA ATG TCT ACT
Gly Ser Cys Gln Thr Thr Tyr Asn Gln Asn Ala Met Phe Pro Gln Leu Ile Met Ser Thr

          910           920           930           940           950           960
           *             •             •             •             *             *
TAT TAT AAT TAC ATG GTT AAT CTT GGT GAT CTA TTT GAA GGA TTC TGA TCA TAA ACA TAA
Tyr Tyr Asn Tyr Met Val Asn Leu Gly Asp Leu Phe Glu Gly Phe ---

          970           980           990          1000          1010          1020
           •             •             •             *             *             *
TAA GGA GTA TAT ATA TAT TAC TCC AAC TAT ATT ATA AAG CTT AAA TAA GAG GCC GTG TTA

          1030          1040          1050          1060          1070          1080
           *             *             *             *             *             •
ATT AGT ACT TGT TGC CTT TTG CTT TAT GGT GTT GTT TAT TAT GCC TTG TAT GCT TGT AAT

          1090          1100          1110          1120          1130          1140
           *             *             *             *             *             •
ATT ATC TAG AGA ACA AGA TGT ACT GTG TAA TAG TCT TGT TTG AAA TAA AAC TTC CAA TTA

          1150
           •
TGA TGC AAGGTACC A GGATCC
          KpnI     BamHI
```

Fig.16. Sequence of pokeweed antiviral protein gene (continued).

(B) Constitutive and Inducible Expression of the full-length *PAP* Gene in *E. coli*

A 1.1 kb cDNA fragment encoding the entire PAP gene, including the N-terminal leader signal, the mature PAP and the C-terminal extra-peptide, as well as a 3' untranslated region was released from pBS$^+$-PAP$_{anti}$ by digestion with *Bam*HI and then cloned in the *Bam*HI site of the modified *p*BR322-based expression vectors, *p*P$_t$SD as a constitutive vector and pP$_t$O$_t$SD as an inducible vector (Ivanov *et al.*, 1987a, 1987b and 1990) (Fig.9 and Fig.17). In order to clone the PAP gene in these expression vectors, both vectors were modified by replacing the fragment between *Xho*I and *Bam*HI sites (located immediately upstream of the PAP gene) with a synthetic DNA duplex containing a consensus Shine-Dalgarno (SD) ribosome-binding site (Fig.17). Cloning of the PAP open reading frame in the sense orientation was confirmed by nucleotide sequencing and was found to be identical (no mutation) to the PAP sequence cloned in plasmid pBS$^+$PAP$_{anti}$ in the antisense orientation (Fig.16).

The three *E. coli* strains used were equally productive when transformed with *p*P$_t$SD-PAP$_f$ (Table 3). As expected, the level of PAP expressed from the inducible construct *p*P$_t$O$_t$SD-PAP$_f$ was dependent on the presence of IPTG in the medium when *E. coli* JM109 and *E. coli* 71-18 (overproducing *lacI*^{q} repressor) were used but was independent of IPTG when expressed in *E. coli* LE392. The low level of PAP$_f$ expression in the first two strains in the absence of IPTG confirmed the previously observed leakage of the *lac* promoter (Ivanov *et al.*, 1990; Chen *et al.*, 1993). In this study, the derepression of the *lac* promoter by IPTG led to a 3-4 fold increase in the PAP yield, twice as high as that observed by Chen *et al.* (1993) using the commercial vector pKK233-2 in *E. coli* JM109. The total yield of PAP in the presence of IPTG was also twice as high which could be explained by the stronger promoter and SD sequence in *p*P$_t$O$_t$SD-PAP$_f$ as

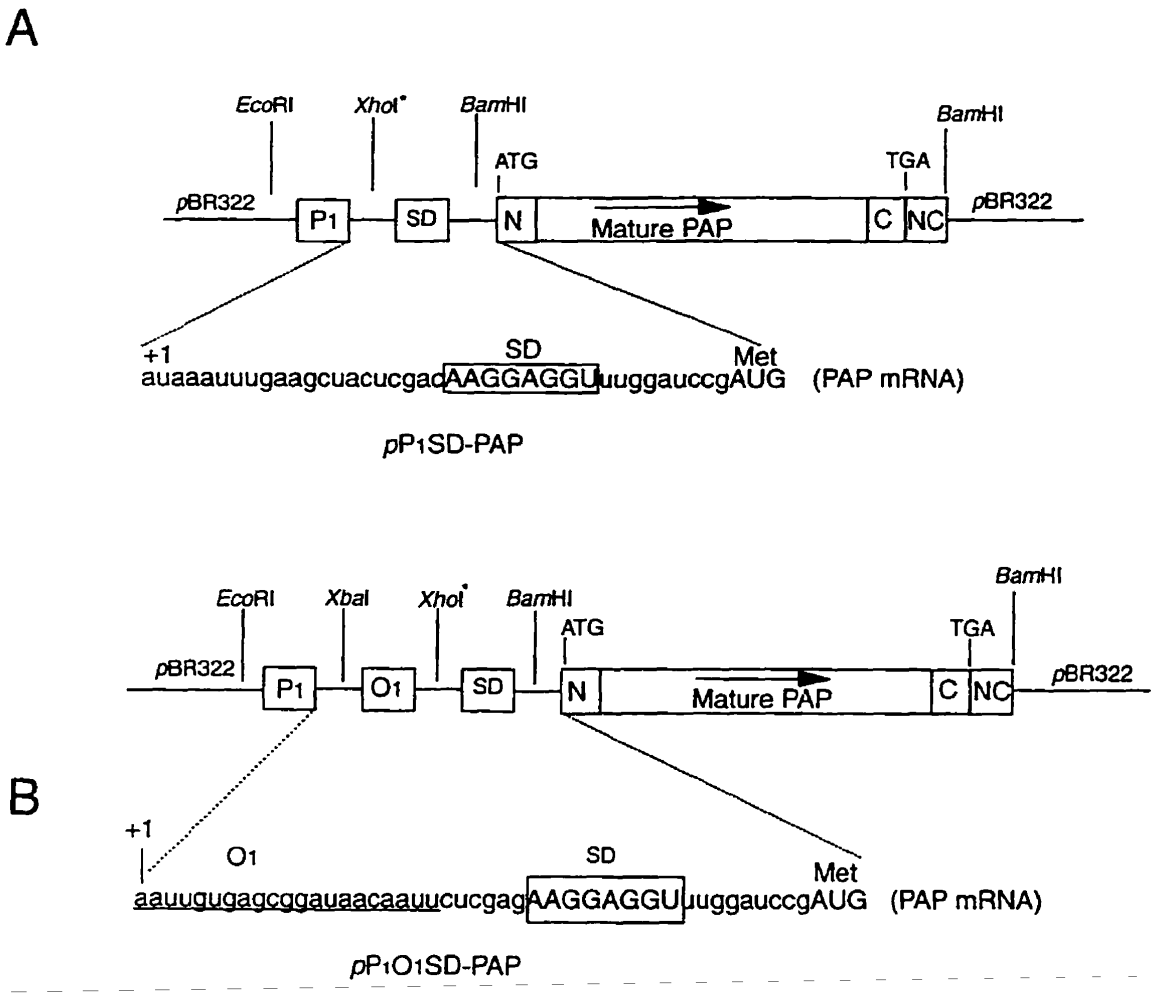


Fig.17. Schematic diagram of vectors for constitutive (A) and inducible (B) expression of the *PAP*$_f$ gene in *E. coli*. Expression vectors are based on the cloning plasmid pBR322. Three (A) or four (B) successive elements are inserted after the *Eco*RI site: P_1, promoter (a synthetic analog of the bacteriophage T5 early promoter); O_1, *lac* operator; SD, Shine-Dalgarno consensus sequence and the PAP gene. In order to insert the PAP gene, two expression vectors (pP_1R_9 and $pP_1O_1R_9$) were modified and a *Bam*HI site was included. The *Bam*HI site was then used for the insertion of the full-length PAP gene. N and C, PAP N- and C-terminal signal peptides; NC, non-coding region of PAP gene. Nucleotide sequences shown represent the 5'-untranslated regions in transcribed mRNAs; +1, the first nucleotide in mRNA; SD consensus sequence is boxed and *lac* operator sequence is underlined.* This *Xho*I site is destroyed after ligation.

Table 3. Yield of recombinant PAP_f and mRNA in *E. coli* strains transformed with PAP_f expression plasmids

Expression plasmid	*E. coli* strain	- IPTG		+ IPTG		+ IPTG Ratios[a]		
		Protein (mg/l)	mRNA (cpm)	Protein (mg/l)	mRNA (cpm)	Protein (mg/l)	mRNA (cpm)	Protein/ mRNA
*p*P$_{I}$O$_{I}$SD	JM 109	0.22	850	0.72	3350	0.10	0.60	0.17
	71-18	0.26	620	0.86	3500	0.12	0.62	0.19
	LE 392	1.10	4050	1.22	3850	0.17	0.69	0.25
*p*P$_{I}$SD	JM 109	6.65	5200	6.78	5350	0.94	0.95	1.00
	71-18	6.80	5780	7.10	5820	0.98	1.04	0.94
	LE 392	7.40	5840	7.22	5620	1.00	1.00	1.00

The yield of recombinant PAP was measured by ELISA (Sambrook *et al.*, 1989) and that of its mRNA was measured by radioactive probe hybridization according to Ivanov and Gigova (1987). IPTG, *lac* operon inducer; PAPf, full-length PAP; *p*P$_{I}$O$_{I}$SD, *lac* operon inducible vector; *p*P$_{I}$SD, constitutive vector.

[a] Ratios relative to that found in pP$_{I}$SD in LE 392.

compared with that in *p*KK233-2. However, the yield obtained with the constitutive vector was 6-10 times higher than that with the inducible promoter (Table 3). To determine the reason for the different yields obtained with the two vectors, the level and stability (half-life) of PAP_f mRNA were also analysed. The content of PAP_f mRNA in all three *E. coli* strains transformed with $pP_IO_ISD\text{-}PAP_f$ did not exceed 60-70% of that obtained with $pP_ISD\text{-}PAP_f$ even in the presence of an excess of IPTG (Table 3). The stability of mRNA from both vectors in the three *E. coli* strains was determined (Ivanov *et al.*, 1992) and found to be almost the same, showing a half-life between 65 and 70 seconds (Data from Dr. Ivan Ivanov). When the protein yield was normalized to the content of mRNA, the PAP_f mRNA transcribed from the inducible plasmid ($pP_IO_ISD\text{-}PAP_f$) was translated at 20-25% the efficiency compared to that when transcribed with the constitutive ($pP_ISD\text{-}PAP_f$) plasmid (Table 3).

The secondary structures of PAP_f mRNA obtained from both $pP_ISD\text{-}PAP_f$ and $pP_IO_ISD\text{-}PAP_f$ were analysed. The secondary structures of the first 50, 100 and 200 nt at the 5'-termini

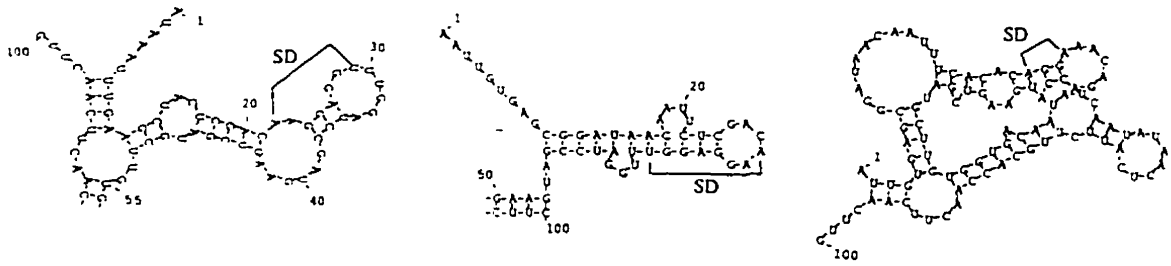


Fig.18. Secondary structures of 5'- termini of PAP_f mRNAs transcribed from different expression vectors: (A) $pP_ISD\text{-}PAP_f$; (B) $pP_IO_ISD\text{-}PAP_f$; (C) *p*KK233-2. SD sequence is marked by brackets. Secondary structures with 50 and 200 nt were not shown. Note: the consensus SD sequence in pKK233-2 is shorter (AGGA) than the commonly used one (AAGGAGGU).

of the two PAP mRNAs (with or without a *lac* operator sequence) were determined using the computer program DNASIS. The SD sequence in mRNA containing a *lac* operator region (Fig.18B and 18C) was found to be involved in a stable secondary structure, whereas the SD sequence in the mRNA obtained from the constitutive plasmid pP$_t$SD-PAP$_f$ was more accessible for RNA-RNA interaction (Fig.18A).

(C) Localization of Expressed PAP in *E. coli*

To determine the location of the full-length PAP in *E. coli*, ultrastructural immuno-gold labelling of PAP was performed. Gold particles were located in clusters in the form of inclusion bodies within the cytoplasm of the cells expressing PAP (Fig.19A, Table 4). Gold particles were also found in low abundance within the cell wall and the periplasm of cells expressing PAP. Very few gold particles (background) were seen in cells harbouring the PAP gene in the antisense orientation (Fig.19B).

Table 4. Distribution of immuno-gold particles in *E. coli* cells expressing the *PAP$_f$* gene

Location	Inclusion body	Cell wall	Cytoplasm (other than inclusion body)
Number of gold particles/cell	11.8 ± 4.3	2.1 ± 1.8	0.8 ± 1.2

Among 100 *E. coli* cells examined, about 25% of cells had inclusion bodies. The number of background gold particles remained low and consistent for cells expressing PAP protein (0.8 gold particles/cell) as well as those not expressing PAP protein (0.9 gold particles/cell). Data derived from 12 different cells containing inclusion bodies. The background data were obtained from 12 cells which did not contain the PAP gene.

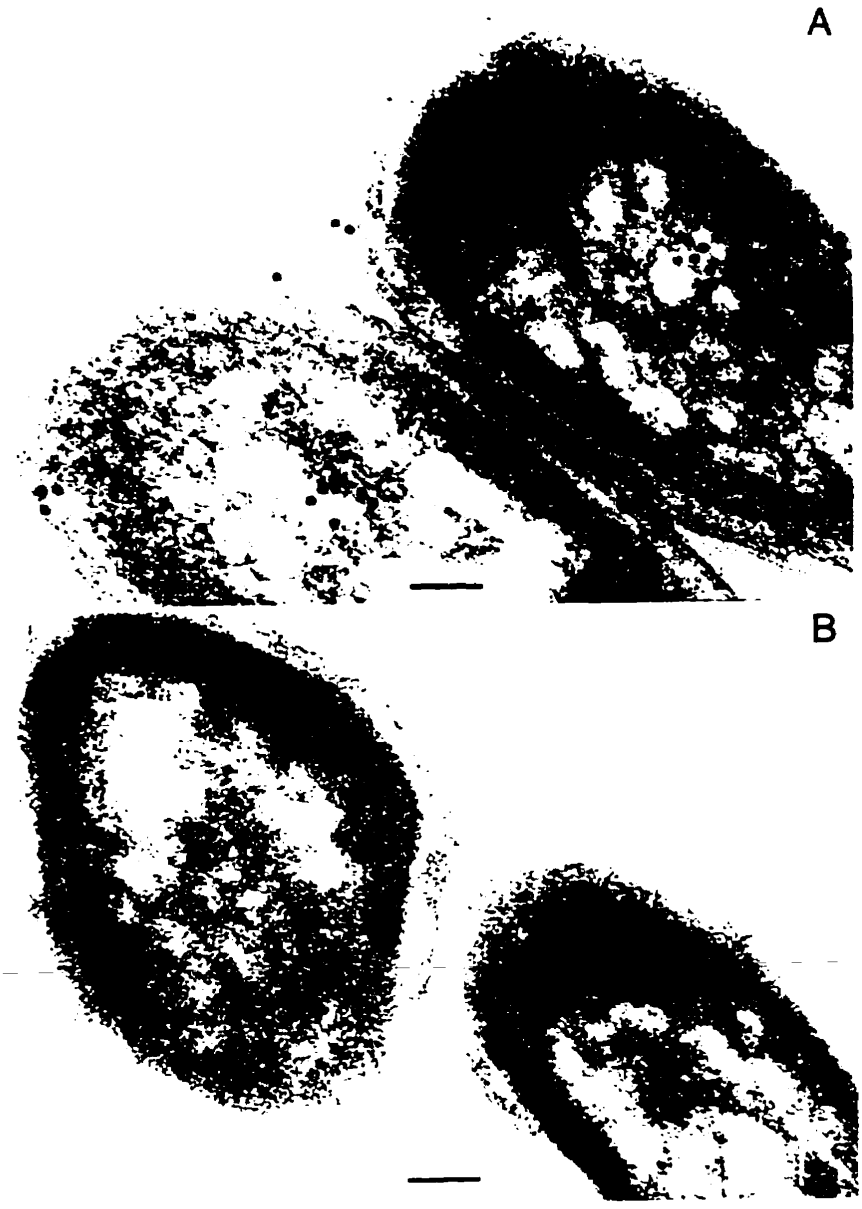


Fig.19. Ultrastructural immuno-gold labelling of *E. coli* cells containing expression plasmids with the full-length PAP gene in the sense (A) or antisense (B) orientation. (A), Gold particles were located in clusters in the form of inclusion bodies in the cytoplasm as well as in low abundance within the cell wall and the periplasm of cells expressing PAP. (B), Very few gold particles could be seen in cells harbouring the PAP gene in the antisense orientation. Bar represents 75 nm.

The concentration of the full-length PAP (PAP_f) in the culture medium was measured by ammonium sulfate precipitation followed by Western blot analysis (Fig.20, lane 2). Both ELISA and dot-blot analyses showed that the concentration of PAP_f in the culture medium did not exceed 2% of total protein. In addition, no more than 5 - 10% of PAP_f was found in the cell periplasm after a mild osmotic lysis. Thus, protein analysis and ultrastructural data indicated that the recombinant PAP_f had an intracellular cytoplasmic localization.

The PAP_f protein expressed in *E. coli* generated two forms in approximately equimolar amount (Fig.20, lane 3). it seems that the molecular weight of the short one is similar to that of the mature PAP. Two forms of PAP but with different molecular weights were also observed in the protein from the cell culture medium (Fig.20, lane 2). However, only one form of PAP was isolated by mild osmotic lysis in the periplasmic space (Fig.20, lane 1).

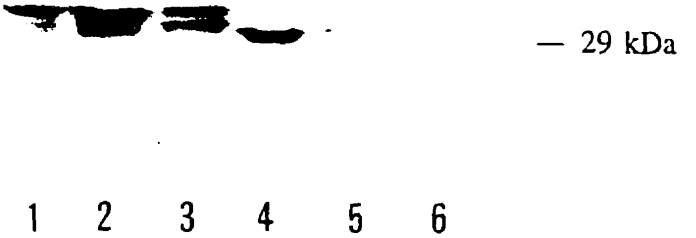


Fig.20. Western blot analysis of the full-length PAP produced by *E. coli* cells. *E. coli* LE 392 cells transformed with $pP_ISD\text{-}PAP_f$ (lanes 1-3) or *E. coli* JM 109 cells (lanes 5-6) transformed with $pP_IO_ISD\text{-}PAP_f$ were cultivated as described in *Materials and Methods*. Total proteins were obtained from equal amounts of precipitated cells (lanes 3, 5 and 6), bacterial periplasm after a mild osmotic shock (lane 1) and culture medium (lane 2). *E. coli* JM 109 cells were cultivated either in the absence (lane 5) or in the presence of IPTG (lane 6). Samples were electrophoresed in a 12.5% SDS-PAGE gel in the presence of native PAP (lane 4), transferred onto a nitrocellulose membrane and treated with PAP specific antibodies. Note: a weak PAP band was visible after IPTG induction in lane 6 but did not photograph well.

(D) Biological Activity Assay of the Full-length PAP Expressed in *E. coli*

The full-length PAP extracted from *E. coli* LE392 cells was tested for its antiviral activity using a local lesion assay on tobacco leaves (Table 5). A mixture of purified tobacco mosaic virus (TMV) (25 μg/ml) and an *E. coli* lysate containing PAP at concentrations as low as 62.5 ng/ml, resulted in a 58% inhibition of viral infection. When the concentration of *E. coli*-expressed PAP was increased to 1 and 4 μg/ml, the inhibition of viral infection increased to 97 and 100%, respectively (Table 5). Comparison of antiviral activities of PAP from pokeweed and PAP from *E. coli* cells was carried out. To eliminate the effect of *E. coli* lysates on the antiviral activity assay, *E. coli* lysate total proteins (100 μg) which do not express PAP were used and the *E. coli* cells containing the vector but devoid of the PAP gene were used as a control. The antiviral activity of PAP derived from *E. coli* lysates was about 70% compared to the commercial PAP extracted from pokeweed plants (Table 5).

The ability of PAP expressed in *E. coli* to inactivate ribosomes and inhibit translation was confirmed in an *in vitro* translation inhibition assay. Wheat germ extracts were pre-incubated with lysates from *E. coli* with or without PAP gene expression. The extent of protein synthesis (ribosome inactivation) was determined by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig.21). Levels of *E. coli* expressed PAP as low as 10 pg were capable of inactivating ribosomes and inhibiting translation (Fig.21, lane 3). No inhibition of translation was observed when similar amounts of protein extracted from *E. coli* transformed with pP$_1$SD which does not express PAP were added to the wheat germ extract (Fig.21, lane 1).

Table 5. Inhibition of Tobacco Mosaic Virus Infection by Recombinant and Natural Pokeweed Antiviral Protein

PAP (μg/ml)	Number of local lesions		Viral inhibition (%)
	Extracts from LE392 with pP_tSD-PAP[a]	Extracts from LE392 without pP_tSD-PAP[b]	
8.0000	0	79 ± 30	100
4.0000	0	82 ± 26	100
2.0000	2 ± 1	83 ± 55	98
1.0000	3 ± 2	100 ± 28	97
0.5000	9 ± 5	104 ± 50	92
0.2500	17 ± 7	114 ± 49	85
0.1250	36 ± 17	110 ± 16	68
0.1250*	10 ± 4	-	91
0.0625	61 ± 17	143 ± 48	58
0.0625*	25 ± 10	-	83
0.0000	174 ± 46	174 ± 46	0

[a] Partially purified cell lysate (see Materials and Methods) from *E. coli* LE392 transformed with the pP_tSD-PAP_f was serially diluted with a phosphate buffer (0.1 M, pH 7.2).

[b] Partially purified cell lysate from *E. coli* LE392 transformed with *p*BR322 was serially diluted with a phosphate buffer (0.1 M, pH 7.2) and used as a control.

* Commercial mature PAP from *Phytolacca americana* (Calbiochem).

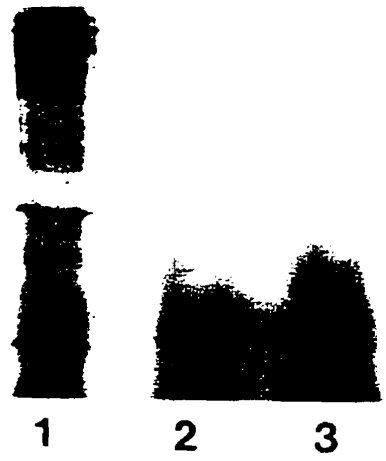


Fig.21. Inhibition of *in vitro* translation by PAP. Prior to addition of BMV RNA templates, wheat germ extracts (Promega) were incubated for 20 min at room temperature with lysates of *E. coli* which expressed or did not express the PAP gene. [^{35}S]-methionine labelled translation products were subjected to electrophoresis and autoradiography. Lane 1 contains 80 ng of *E. coli* lysate containing pP$_1$SD (without the PAP gene); Lane 2 contains 80 ng of *E. coli* lysate containing pP$_1$SD plus 10 pg of purified PAP from pokeweed plants; Lane 3 contains 80 ng of *E. coli* lysate expressing 10 pg of PAP protein (pP$_1$SD-PAP).

II Mutational Analysis of the PAP Gene in *E. coli*

(A) Effect of the N-terminal signal peptide deletion

The cloning strategy and structure of the plasmids for expression of the N-terminus deleted PAP gene in *E. coli* were similar to those described in Fig.9 and Fig.17.

The 5'-terminus of the PAP gene was subjected to deletion mutagenesis in order to study the significance of the signal peptide on the toxicity to *E. coli* and on the antiviral activity. When the PCR-derived deletion of the PAP gene ($PAP_{\Delta N}22$) was cloned, 22 out of 52 clones contained the N-terminal signal deleted PAP gene in sense orientation but only four clones produced a detectable level of PAP by Western blot (Table 6).

Table 6. Cloning of the N-terminal signal-deleted PAP gene ($PAP_{\Delta N}22$) in the expression vector pP_1SD

PAP gene construct clones	5'terminal codons removed	Number of colonies containing PAP gene			Immunoreactive clones in sense colonies	
		Total	Sense	Antisense	number	percentage
PAP_f	0	44	21	23	21	100
$PAP_{\Delta N}22$	22	52	22	30	4	18.2

The following clones (producing immunoreactive PAP protein or not) were chosen for further analysis: 1. *PAP_f* (carrying the full-length PAP gene); 2. four clones from the PCR block deletion group $PAP_{\Delta N}22$ ($PAP_{\Delta N}22$a-d). DNA sequencing showed that only the full-length PAP gene (PAP_f) had the correct primary structure (Table 7). Deletion of the 22 amino acid signal peptide resulted in the production of a very small number of colonies. Among 22 clones containing the PAP in the sense orientation, only 4 clones produced PAP but at lower levels. The other 18 clones did not produce any detectable PAP. Analysis of 4 clones (two producing and another two not producing PAP) revealed that all these clones contained mutations (Table 7). Two clones which produced an immunoreactive PAP protein contained a six-nucleotide T^{205}ACAAA insertion after A^{204} ($PAP_{\Delta N}22$-a) or a large insertion after A^{652} ($PAP_{\Delta N}22$-b), while the other two clones which did not produce an immunoreactive PAP protein contained one nucleotide deletion at G^{21} ($PAP_{\Delta N}22$-c) or a large insertion (more than 170 nucleotides) at T^{225} ($PAP_{\Delta N}22$-d) (Table 7).

Expression vectors containing the full-length or truncated forms of the PAP gene were transformed into *E. coli* LE392 cells and the production of PAP was visualized by Western blotting and quantitated by dot-blot analysis. Western blotting of the proteins from the constructs producing immunoreactive PAP showed a band with similar M_r compared to the full-length PAP (Fig.22, lanes 1 and 3). Data presented in Table 7 indicate that the yield of PAP obtained from the mutant constructs $PAP_{\Delta N}22$(a,b) was much lower than that of the full-length PAP gene.

Antiviral activity of the proteins coded by the PAP gene constructs described above was tested by a local lesion assay using tobacco plants and tobacco mosaic virus (TMV). When the TMV samples were pre-treated with lysates of *E. coli* cells transformed with the full-length PAP

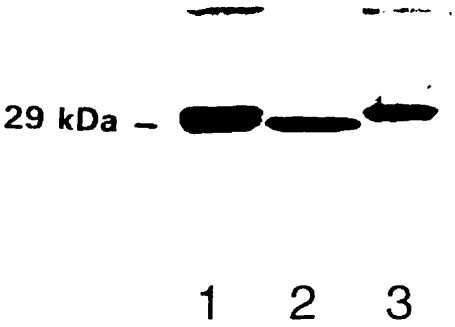


Fig 22. Western blot analysis of total proteins from *E. coli* cells expressing the N-terminal signal-deleted PAP gene. *E. coli* cells were lysed and subjected to Western blot analysis. Lane 1, total proteins (100 μg) from *E. coli* cells transformed with plasmid pP_1SD-PAP containing the full-length PAP gene in sense orientation; lane 2, commercial PAP (0.5 μg) purified from pokeweed plants (without the 22 aa N-terminal signal peptide and the C-terminal extension); lane 3, total proteins (200 μg) from *E. coli* cells transformed with plasmid $PAP_{\Delta N}22$-a without the N-terminal signal codons.

construct, a 100% reduction in the number of local lesions was observed (Table 7). Two of the clones belonging to the $PAP_{\Delta N}22$ deletion series ($PAP_{\Delta N}22$-a and $PAP_{\Delta N}22$-b) also had mutations in their nucleotide sequences. Their antiviral activity ranged from 53% to 59% of that of the full-length PAP although the same amount of PAP was used (Table 7).

Table 7. Effect of the N-terminal signal peptide deletion on the antiviral activity of PAP

PAP gene construct[a]	Yield of PAP (% of total *E. coli* proteins)	TMV Antiviral activity (% inhibition)[b]	PAP gene structure
PAP_f	1.24	100	No mutation
$PAP_{\Delta N}22$-a	0.12	59	$T^{205}ACAAA$ insertion
$PAP_{\Delta N}22$-b	0.08	53	Large insertion after A^{652}
$PAP_{\Delta N}22$-c	0	ND	Deletion of G^{21}
$PAP_{\Delta N}22$-d	0	ND	Large insertion after T^{225}

[a] $_{\Delta N}22$ represents the N-terminal signal peptide codons (22) deleted, while the letters from a to d after the $_{\Delta N}22$ designate the clone number which was selected for study. ND = not determined.

[b] Same PAP concentration (0.125 μg/ml) based on Western dot-blot was used for each construct.

(B) Effect of the C-terminus deletion

To understand the role of C-terminal region, 62 codons from the C-terminus of the full-length PAP gene were removed. Two unique restriction sites (*Xho*I located in the C-terminus and *Xba*I near the end of the 3' untranslated region of the full-length PAP gene) were selected (Fig.23). Thus, the synthetic DNA duplex cassette containing two stop codons (TGATGA) and a *Bgl*II site (for screening) was directionally ligated into the plasmid pP_tSD-PAP linearized by

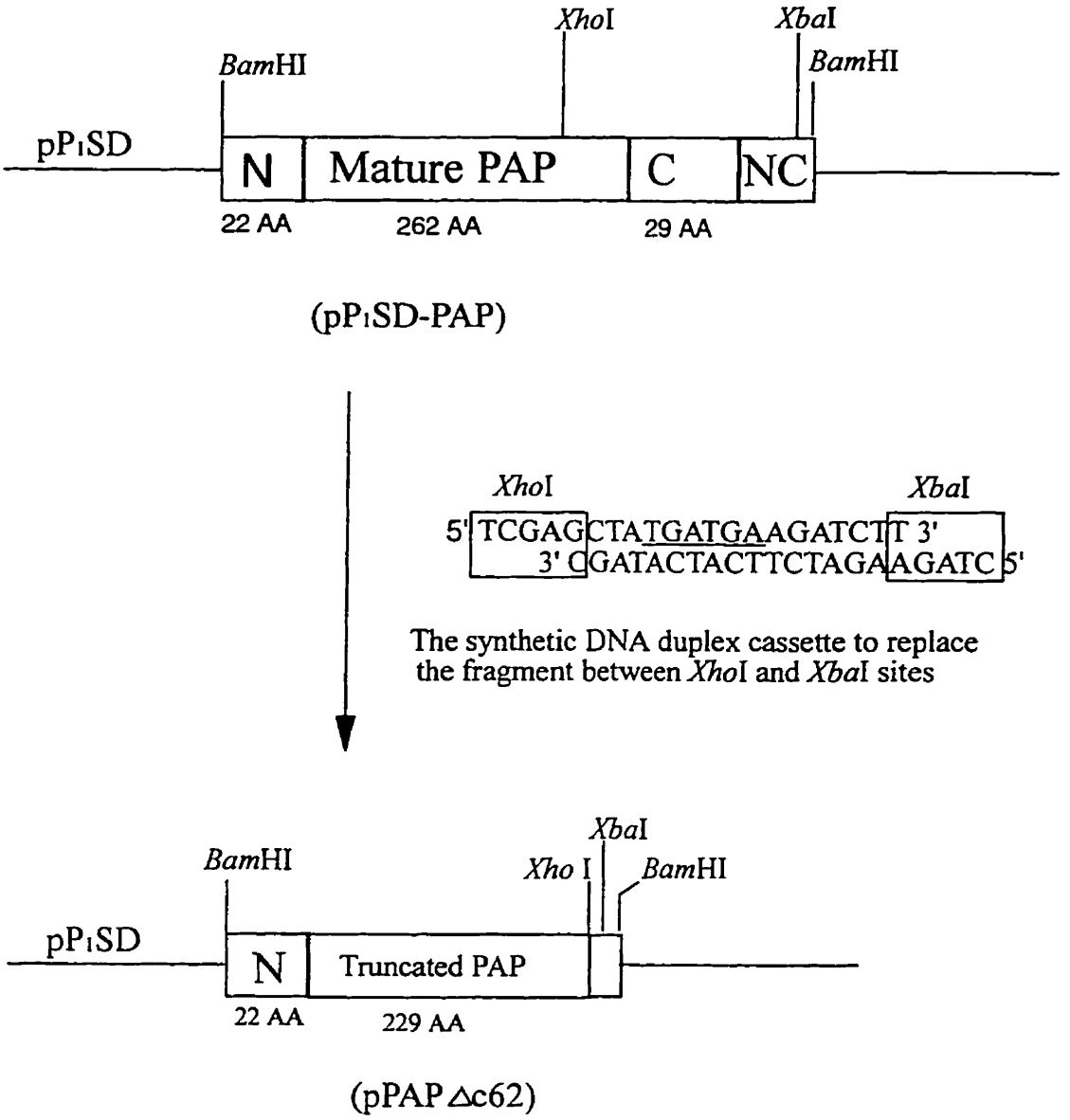


Fig.23. Construction of the C-terminal deleted PAP gene in the expression vector pP$_i$SD-PAP. A synthetic DNA duplex containing *Bgl*II (screening marker), two stop codons (underlined TGATGA) and sticky *Xho*I and *Xba*I overhangs on both termini (boxed) was used to replace the original fragment between *Xho*I and *Xba*I of the expression vector pP$_i$SD-PAP, resulting in a truncation of 62 amino acids from the C-terminus. N and C, PAP N- and C-terminal signal peptides; NC, non-coding region of the PAP gene.

Fig.24. Western blot analysis of C-terminal truncated form of PAP. Lane 1, Commercial PAP from pokeweed plants; lanes 2 - 4, *E. coli* lysate total proteins (200 μg) containing different expression vectors; lane 2, pP_ISD (devoid of PAP gene); lane 3, pP_ISD-PAP (full-length PAP gene); lane 4, $pPAP_{\Delta C}62$ (C-terminus truncated form of the PAP gene.

*Xho*I and *Xba*I. After transformation, 216 colonies appeared while the background (same ligation reaction but devoid of the DNA cassette) had only 22 colonies. Plasmid DNA from twelve colonies was then prepared and digested with *Pst*I (located in the vector) and *Bgl*II (located in the PAP insert). Eight of these contained the correct fragment size after digestion. One of the clones ($pPAP_{\Delta C}62$) was further confirmed by digestion with different combinations of restriction enzymes *Pst*I+*Xba*I, *Pst*I+*Xho*I. Western dot blot analysis showed that the truncated form of the PAP was expressed (Fig.24) but the yield was much lower (0.35 mg/L) than that of the full-

length PAP (4.32 mg/L).

The local lesion assay with tobacco mosaic virus (TMV) showed that the antiviral activity of the C-terminal deleted PAP (Table 8) decreased (from 93% to 28%) and further statistical analysis demonstrated that there was a significant difference in antiviral activity between the C-terminus deleted PAP (clone $pPAP_{\Delta C}62$) and the full-length PAP gene. A significant difference was also observed between the C-terminal deleted PAP and the negative control (devoid of the PAP gene). The C-terminal truncated form of PAP was also tested for its ribosome-inactivating activity. BMV-RNA was used as a template in an *in vitro* wheat germ translation system. As

Table 8. Comparison of antiviral activities between the full-length PAP and the C-terminal deleted form of PAP

	E. coli protein devoid of PAP gene (pP_tSD)	*E. coli* protein with C-terminus deleted PAP gene ($pPAP_{\Delta C}62$)	*E. coli* protein with full-length PAP gene (pP_tSD-PAP)
Average No. of lesions[a]	40 ± 6	29 ± 7	3 ± 1
Percentage of inhibition[b]	0	28[c]	93

[a] data derived from two independent experiments, each consisting of 10 half leaves

[b] *E. coli* cells harbouring vectors containing the full-length PAP (pP_tSD-PAP), C-terminal deleted PAP ($pPAP_{\Delta C}62$) and devoid of PAP gene (pP_tSD) were cultured, sonicated and assayed.

[c] Same concentration of PAP (1 μg/ml) based on Western dot-blot was used for both the full-length and the C-terminal deleted PAP genes.

shown in Fig.25, the truncated PAP was approximately 10% as active as the full-length PAP, i.e., 1 pg of the truncated PAP resulted in similar *in vitro* translation reaction inhibition (Fig.25, lane 8) compared to that with the 0.1 pg (100 fg) of the full-length PAP (Fig.25, lane 4).

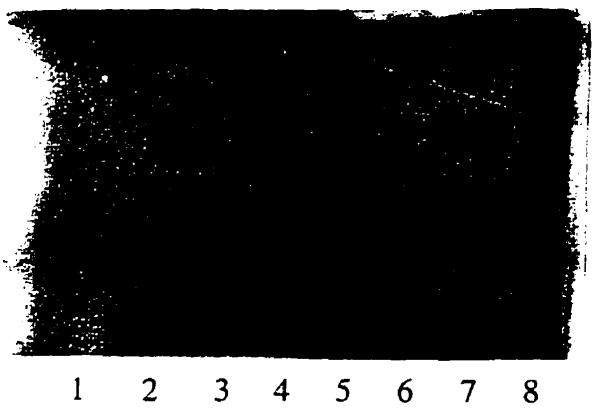


Fig.25. Inhibition of *in vitro* translation by the C-terminal truncated form of PAP. Prior to addition of brome mosaic virus (BMV) RNA template, wheat germ extracts (Promega) were pre-incubated for 20 min at room temperature with lysates of *E. coli* which expressed either the full-length or C-terminal truncated form of the PAP gene. [^{35}S]-methionine labelled translation products were subjected to electrophoresis and auto-radiography. A total of 80 ng of *E. coli* lysate proteins containing various amounts of *E. coli* expressed PAP were added to the reaction mixtures. Lanes: 1 - 5, *E. coli* lysate from the full-length PAP gene (pP_1SD-PAP); lanes: 6-8, *E. coli* lysate from the C-terminus deleted form of the PAP gene ($pPAP_{\Delta C}62$). lane 1, 100 pg; lane 2, 10 pg; lane 3, 1 pg; lane 4, 100 fg; lane 5, 10 fg; lane 6, 100 pg; lane 7, 10 pg; lane 8, 1 pg.

Note: As little as 10 pg of the C-terminus deleted form of PAP (lane 7) inactivated the *in vitro* translation.

III. High Level Expression of PAP Using Non-Shine-Dalgarno Translational Initiators

(A) PL, a polylinker sequence from plasmid pP_l-PL

(i) High level expression of PAP without the Shine-Dalgarno sequence

The full-length PAP gene released after digestion with *Bam*HI from plasmid pBS^+-PAP_{anti} was cloned in the *Bgl*II site of a short polylinker sequence (containing *Xho*I, *Bgl*II, *Xba*I, *Eco*RV and *Hind*III) placed downstream of the P_l promoter in plasmid pP_lPL (Fig.26A). This construct was used as a negative control and was not expected to produce PAP due to the lack of consensus SD sequence. Surprisingly, this construct (pP_lPL-PAP) was found to be active. Moreover, the level of expression of the PAP gene from this construct was even higher than that obtained with the SD consensus sequence (Table 9 and Fig.28). As seen in Fig.26B, there is no typical SD sequence in the 5' non-translated region preceding the initiation codon (except for the two triplets GAG and AGA situated at -12 to -10 and -7 to -5 nt before the ATG codon).

(ii) Complementarity of the 5' terminal region of PAP mRNA to the 16S ribosomal RNA of E. coli

To determine why the PAP mRNA from the pP_lPL-PAP clone devoid of a SD sequence, is translated in *E. coli* cells, a search for complementarity between the 5' terminal un- translated region of the PAP mRNA and the *E. coli* 16S rRNA was carried out using the program SEQAID. As shown in Figs. 26B and 27, two boxes of 7 nt each, were found to be complementary to two distinct areas in the 16S rRNA. Box I is complementary to nucleotides 1434-1440 and box II to 507-513. To determine whether the observed complementarity was coincidental or related to the initiation of translation, the two boxes were mutated as shown in Fig.26B and the protein yield

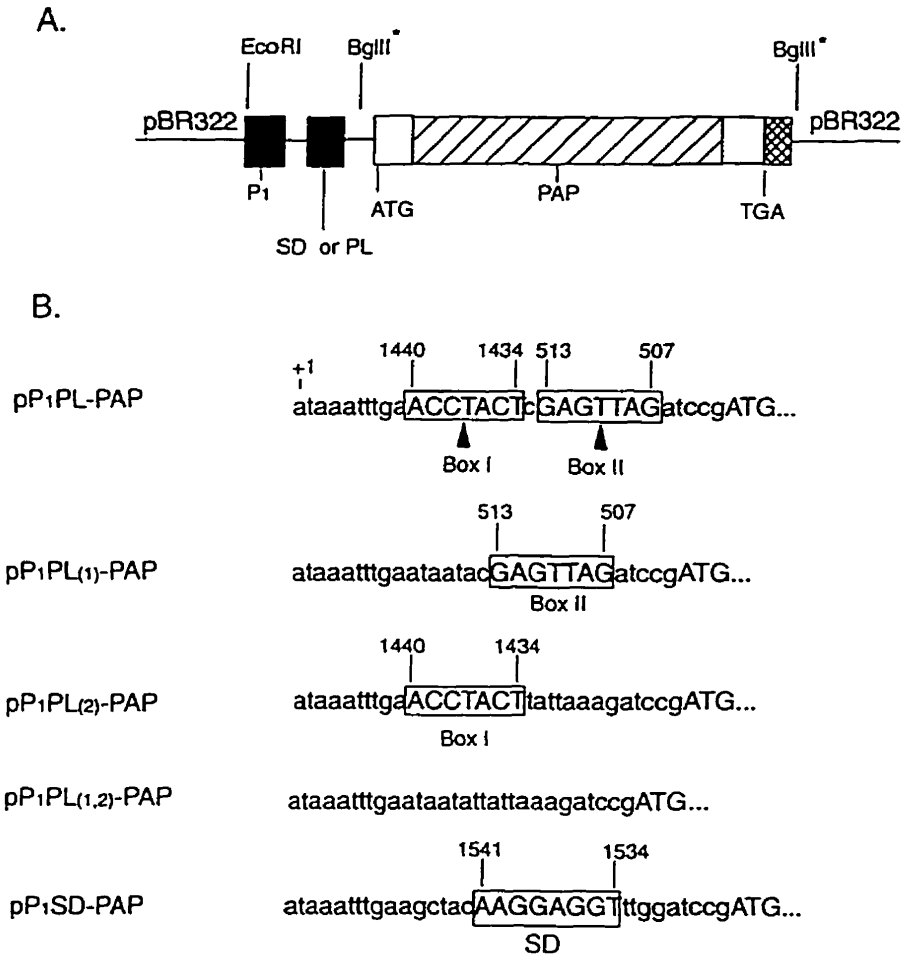


Fig.26. Construction of the full-length PAP gene in a non-SD expression vector using a PL sequence from a polylinker of plasmid pP_1-PL.

A. General structure of expression plasmids. Expression plasmids were pBR322 based. Three successive elements were inserted between the *Eco*RI and the newly introduced *Bgl*II sites: P_1, promoter (a synthetic analog of the bacteriophage T5 early promoter); SD, Shine-Dalgarno consensus sequence (AAGGAGGT); PL, an *Xho*I-*Bgl*II portion of a polylinker containing the cloning sites *Xho*I, *Bgl*II, *Xba*I, *Eco*RV and *Hin*dIII (ACCTACTCGAGTTAG). Hatched box: coding region corresponding to the mature PAP; open boxes: coding regions for PAP N- and C-terminal signal peptides; cross-hatched box: non-coding region of PAP gene. *BglII is compatible with *Bam*HI but not recleavable after ligation.

B. Nucleotide sequences of the 5' non-coding regions of PAP mRNA constructs. Numbers above sequences represent the position of regions complementary to 16S rRNA. +1: the first nucleotide in mRNA transcribed from the P_1 promoter.

Table 9. Yield of the full-length PAP mRNA and full-length PAP expressed in *E. coli* cells

Construct[a]	PAP mRNA[b] (cpm)	Relative yield of PAP mRNA[c]	PAP[d] (mg/l)	Relative yield of PAP[e]
pP_1SD-PAP	2950	1.0	2.1	1.00
pP_1PL-PAP (Wild-type)	2850	0.96	4.8	2.30
$pP_1PL_{(1)}$-PAP (Box I mutated)	3000	1.02	1.8	0.86
$pP_1PL_{(2)}$-PAP (Box II mutated)	2780	0.94	0.9	0.42
$pP_1PL_{(1,2)}$-PAP (Both boxes mutated)	2880	0.98	0.7	0.33
pP_1PL-PAP_{anti} (antisense)	90	0.03	0.0	0
pBR322	60	0.02	0.0	0

[a] Nucleotide sequences of the translation initiation signals are shown in Fig.26.

[b] PAP mRNA yield is based on ^{32}P-radioactivity counting. Data represent average values of three independent experiments.

[c] Yield of PAP mRNA relative to that obtained with the plasmid pP_1SD-PAP (value = 1.0).

[d] Yield of PAP from one litre of bacterial culture as analyzed by ELISA in *Materials & Methods*.

[e] Yield of PAP relative to that obtained with pP_1SD-PAP.

was measured. The results presented in Table 9 and Fig.28 show that the yield of PAP decreased 2.7 and 5.5 times with respect to pP_1PL-PAP respectively, when box I or box II was made non-complementary to the corresponding sites in 16S rRNA. To estimate the baseline level of PAP expression, a new construct was prepared in which both box I and box II were mutated (Fig.26B). Analysis of this new construct by ELISA showed that the yield of PAP expressed was 0.7 mg/litre, i.e., slightly less than that with the box I alone (Table 9). Since mutation of either box I or box II decreased the expression of PAP, I postulated that either box I or box II is capable of initiating translation (though with different efficiencies) of PAP mRNA in *E. coli* cells. In addition, the fact that both box I and box II are required for the maximal expression indicates that the two boxes act synergistically in the translation initiation process.

The free energy of the hypothetical interaction was calculated. Values of -1.2 and -2.4 Kcal/mole for AT and GC pairs were assigned to AT and GC pairs, respectively (Tinoco *et al.*, 1971). The free energy of binding for each of the two boxes to 16S rRNA was estimated to be $\Delta G = -10.8$ Kcal/mol each, a value which was very close to that of the consensus S/D sequence ($\Delta G = -12.8$ Kcal/mol) (Fig.29C).

(iii) Other factors affecting the initiation of translation of PAP in E. coli

In addition to the two upstream boxes described above (relative to AUG start codon), two downstream boxes were also found in the coding region of PAP mRNA, at positions +31 to +40 (box III) and +73 to +80 (box IV). These were found to be complementary to the nucleotide domains 541-548 (estimated $\Delta G = -14.0$ Kcal/mol) and 1320-1329 (estimated $\Delta G = -14.8$ Kcal/mole) in the 16S rRNA respectively (Fig.29A). It is known that nucleotide changes in the 5' terminal region of mRNA might affect not only translation but the efficiency of transcription as

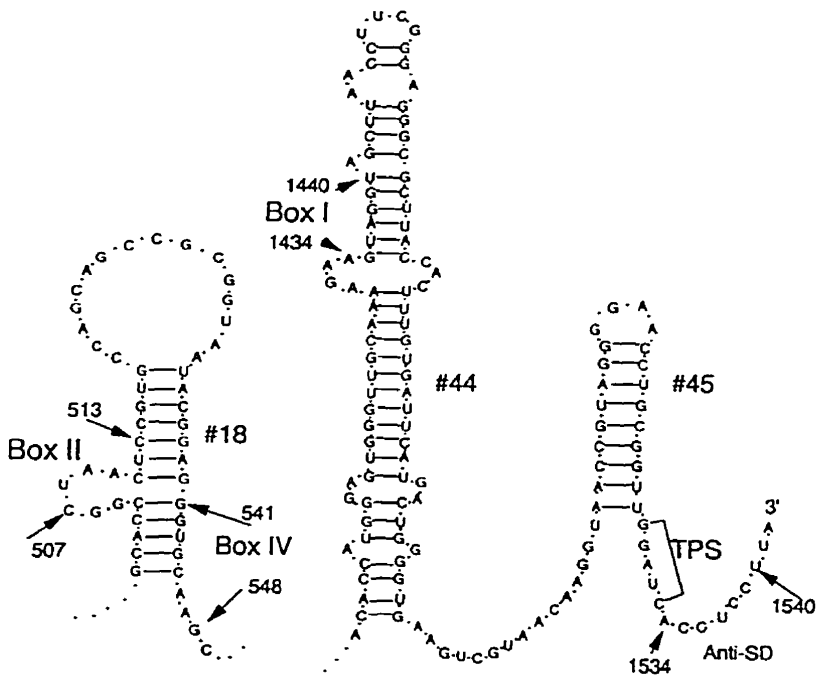


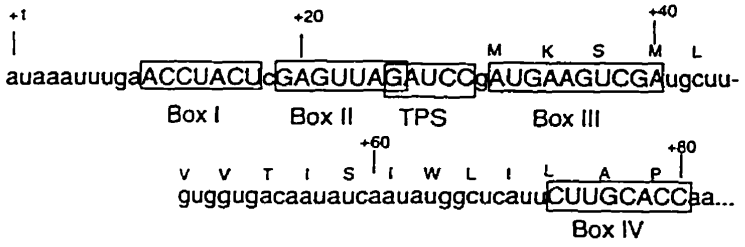
Fig.27. Nucleotide sequences in 16S rRNA complementary to the PL translational initiator. Secondary structures of helical domains #18, #44 and #45 of 16S rRNA are according to Brimacombe *et al.* (1988). Arrows and bracket mark the first and the last nucleotide of regions complementary to box I, box II, box IV, SD and translation-promoting sequence (TPS) (Thanaraj and Pandit, 1989).

well (Helke *et al.*, 1993; Ivanov *et al.*, 1987a, 1987b and 1992). To determine whether the observed differences in the yield of PAP were related to changes in the transcriptional efficiency of mRNA, content of PAP mRNA in the *E. coli* cells was also measured. The yield of PAP mRNA was fairly constant for all the expression plasmids used under the same P_I promoter (Table 9). From these results it could be concluded that the differences in protein yield obtained with the different expression plasmids containing the same PAP gene were related to a difference in efficiency of translation rather than a difference in transcription.

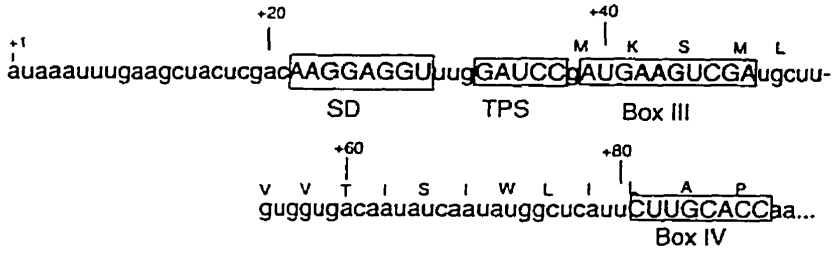
1 2 3 4 5 6 7

Fig.28. Western blot analysis of PAP expressed under wild-type or mutated PL translational initiators in *E. coli*. Lane 1, 0.5 μg of PAP protein purified from pokeweed plant; 2-7, 100 μg of a total protein extract from *E. coli* cells containing the PAP gene in either sense or antisense orientation. Lane 2, pP_ISD-PAP (containing an SD sequence and PAP gene in sense orientation); lane 3, pP_IPL-PAP_{anti} (containing a PL sequence i.e., box I + box II and PAP gene in antisense orientation); lane 4, pP_IPL-PAP (containing a PL sequence and PAP gene in sense orientation); lane 5, $pP_IPL_{(2)}$-PAP (containing box I and the PAP gene as in lane 4); lane 6, $pP_IPL_{(1)}$-PAP (containing box II and the PAP gene as in lane 4); lane 7: $pP_IPL_{(1,2)}$-PAP (devoid of both boxes and the PAP gene as in lane 4).

A (pP_tPL-PAP)



B (pP_tSD-PAP)



C

PAP mRNA sequence	Nucleotides in the 16S rRNA	ΔG (Kcal/mol)
box I	1434-1440	- 10.8
box II	507 - 513	- 10.8
box III	1320 - 1329	- 14.8
box IV	541 - 548	- 14.0
SD	1534-1540	- 12.8
TPS	1529-1533	- 7.6

Fig.29. Nucleotide domains in PAP_t mRNA complementary to different regions in *E. coli* 16S rRNA. Nucleotide sequences: mRNAs transcribed from either PL (A) or SD (B) containing plasmids (pP_tP_L-PAP and pP_tSD-PAP). Boxed nucleotides: domains complementary to regions in 16S rRNA. (C) Coordinates of the complementary regions from A and B in the 16S rRNA molecule. The free energy of the hypothetical interaction is calculated according to Tinoco *et al.* (1971). TPS, translation promoting sequence according to Thanavaj and Pandit (1989).

It has been shown that the secondary structure of mRNA at the 5'-terminal region might be a key factor in determining the efficiency of translation initiation in prokaryotes (Kurland, 1991; Gold, 1988). The secondary structure of PAP mRNA transcribed from all the expression plasmids described above was analyzed by computer software DNASIS. The first 200 or 300 nucleotides at the 5' end of PAP mRNA were predicted to be folded and thus two sets of secondary structures were obtained. The translation initiation signals (box I, box II and SD) were found mostly in single-stranded regions rather than in stable secondary structures (data not shown).

To determine the universality of the PL sequence to initiate translation in *E. coli*, five different eukaryotic genes were also used as reporters. Results indicated that these genes were expressed by the PL sequence as a translational initiator (Ivan Ivanov, personal communication).

(B) Effect of a 33-nucleotide fragment from potato virus X (PVX) on the expression of the PAP gene in *E. coli*

It was found that the sequence upstream of the PVX coat protein gene as a ribosome-binding site functions very well in transgenic plants (Leclerc, 1994). To test whether the same sequence works in prokaryotes such as *E. coli*, an *Xba*I/*Hind*III fragment, containing 33 nucleotides (upstream of the PVX CP gene) and the full-length PAP gene, was released from the binary vector pGA-PAP and cloned into the vector pP_1-PL (devoid of a Shine-Dalgarno sequence) digested with *Xba*I and *Hind*III (Fig.30A). Sixteen clones were selected and restriction digestion of these clones with *Xba*I and *Hind*III showed that 15 clones contained the PVX-PAP fragment and expressed PAP.

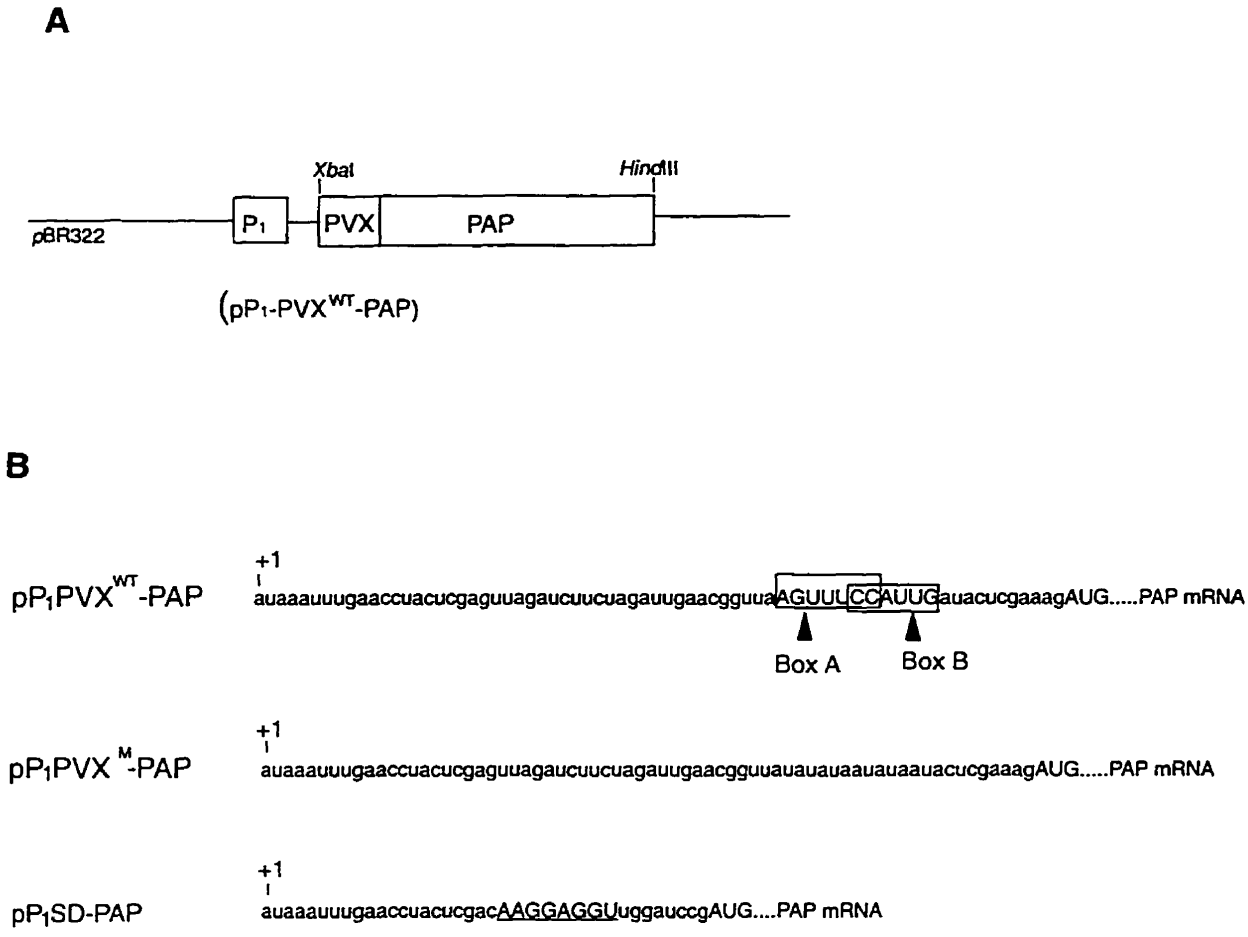


Fig.30. Non-SD expression vector using a 33-nucleotide sequence from PVX. (A). Plasmid construction. Production of plasmid pP_1-PVX^{WT}-PAP is illustrated. (B). Nucleotide sequences of 5'-end non-coding regions of PAP mRNA constructs. pP_1-PVX^{WT}-PAP, containing wild-type PVX sequence; pP_1-PVX^{M}-PAP, containing the PVX sequence with mutations; SD, Shine-Dalgarno consensus sequence.

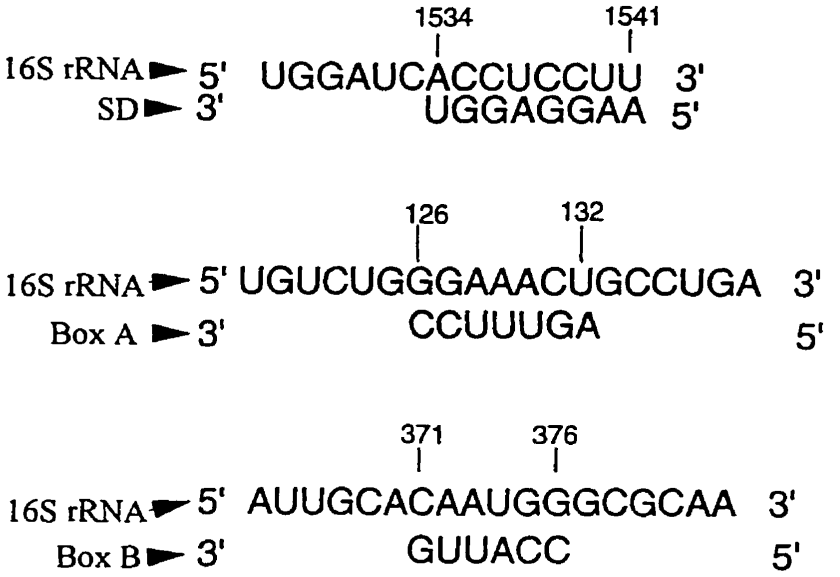


Fig.31. Complementarity between PVX conserved boxes and the 16S rRNA of *E. coli*. 16S rRNA is presented on the upper line. Regions which carry Shine-Dalgarno sequence, or PVX boxes A and B (as in Fig.30) are presented on the lower line.

Two overlapping boxes in the 5' non-translated region of the mRNA which are complementary to the 16S rRNA, were identified by computer analysis (Fig.30B and 32). Box A is complementary to nucleotides 126-132 and box B to 371-376 of the 16S rRNA (Fig.30B and Fig.31).

To confirm whether boxes A and B (Fig.30B) were responsible for the expression, both boxes were mutated by polymerase chain reactions (PCR). An upstream primer from PAP 5' terminus, PM_{PVXF} (5'CTAG**TCTAGA**TTAAACGGTTATAT-ATAATATAATACTCG-AAAGAT-GAAG 3') containing an *Xba*I site (bold) and the mutated boxes (underlined), a downstream

antisense primer, P_{SphI} (5' AGGTTGAGGCCGTTGAGCA 3') complementary to the unique *SphI* region of the template plasmid pP_1-PVX^{WT} were used. The PCR product (1.8 kb) containing the mutated 33 nucleotides from PVX as well as the beginning of the PAP gene, were verified by restriction analysis. Then, the PCR product was digested with *Xba*I and *Hind*III and cloned into the plasmid pP_1-PL linearized by *Xba*I and *Hind*III. Restriction analysis by *Xba*I and *Hind*III and DNA sequencing showed 6 out of 12 colonies contained mutated fragments. PAP gene expression assay (Fig.32) showed that the yield of the construct containing the PVX^{WT} 33 nucleotide sequence was 1.2 mg/l, whereas the yield obtained with the construct containing a Shine-Dalgarno sequence was 4.0 mg/l. As seen from Fig.32, lane 5, the most inefficient expression of the PAP gene was found with the construct containing the PVX^{M} sequence. All 5 clones examined which contained the PVX^{WT} 33 nucleotide sequence revealed similar expression levels.

1 2 3 4 5

Fig.32. Western blot analysis of PAP expressed in *E. coli* under control of a 33 nucleotide fragment from PVX. Preparation of samples is described in *Materials and Methods*. Lane 1, 0.25 μg PAP purified from pokeweed plants. lanes 2 - 5, total protein (100μg) from *E. coli* cells containing different vectors; lane 2, pP_1SD-PAP (PAP gene in sense orientation); lane 3, $pP_1SD\text{-}PAP_{anti}$ (PAP gene in antisense orientation); lane 4, pP_1-PVX^{WT}-PAP; lane 5, pP_1PVX^{M}-PAP (mutated PVX sequence).

IV Development of Transgenic Tobacco Plants Resistant to Viral Infection

(A) Construction and transfer of the full-length PAP gene and its C-terminal truncated forms into *A. tumefaciens*

In order to confer resistance to viral infection by expressing the PAP gene in transgenic plants, it was necessary to incorporate a ribosome binding site upstream of the PAP gene that could be recognized in plants. This was accomplished by inserting a synthetic *Xba*I/*Taq*I cassette containing 33 nt from the upstream region of the PVX coat protein gene and a part of the 5' region of the PAP gene (Fig.10). The chimeric construct was inserted between the CaMV 35S promoter and nopaline synthetase (NOS) polyadenylation terminator within the plant expression vector pGA643 (An *et al.*, 1989) described in Fig.10.

For the C-terminal deleted forms of the PAP gene, three clones containing serial deletions at the C-terminus were digested with *Sac*I/*Sal*I to produce a downstream *Sac*I/*Sal*I fragment (Fig.11). The upstream *Xba*I/*Sac*I fragment containing the ribosome-binding site (RBS) was obtained from plasmid pJ29 (Fig.10) and was ligated with the PAP gene. Thus, the chimeric construct contained the RBS from PVX and truncated forms of the PAP gene. The recombinant pGA643 containing the full-length PAP gene or C-terminal deleted forms of the PAP gene was transformed into competent *E. coli* DH5α. Five colonies from each construct were collected and their plasmid DNA was subjected to restriction analysis (*Hind*III, *Xba*I, *Xba*I+*Acc*I, *Xba*I+*Eco*RI, *Xba*I+*Sac*I for full-length PAP gene and *Hind*III+*Sal*I, *Hind*III+*Xba*I, *Sac*I and *Eco*RI for C-terminal truncated forms of PAP gene). Results showed that all 5 clones with the full-length PAP gene and at least 2 clones from each C-terminus truncated construct contained correct PAP inserts, while others did not have the insert. Five correct clones from the following constructs

designated as pGA, pGA-PAP, pGA-PAPΔ36, pGA-PAPΔ62 and pGA-PAPΔ131 (numbers representing the aa removed from the C-terminus) were then transformed directly into *A. tumefaciens* by $CaCl_2$ and liquid nitrogen methods (Holster *et al.*, 1978). Transformants were selected on YT plates containing kanamycin (30 μg/ml), tetracycline (10 μg/ml) and streptomycin (30 μg/ml). The transformed *A. tumefaciens* colonies were grown in liquid YT medium containing the same concentrations of antibiotics and several clones of the full-length and C-terminal truncated forms of the PAP gene were obtained. Plasmid DNA was verified by restriction analysis.

(B) Transformation and regeneration of transgenic tobacco plants

In order to select transformed tobacco plants (*Nicotiana tabacum* cv. *Xanthi*, a local lesion host for TMV), 50 and 100 μg/ml of kanamycin were tested. It was found that 50 μg/ml kanamycin did not completely kill all of the untransformed tobacco cells (3 shoots were produced from 20 leaf explants) but 100 μg/ml kanamycin killed all untransformed cells, indicating that selection at 100 μg/ml kanamycin was complete. Therefore, 100 μg/ml kanamycin was used throughout the experiment. Transformation of tobacco leaf explants with five different constructs including one (pGA) without the PAP gene (as a control) generated various numbers of shoots. Two controls without the PAP gene were used. One was the wild-type and untransformed tobacco plants (sensitive to kanamycin) and the other was tobacco plants transformed with pGA643 (devoid of the PAP gene). Construct with deletion of 131 codons of the PAP gene produced large numbers of shoots (Table 10). However, the construct with a 62 aa deletion at the C-terminus resulted in a fewer shoots, while the two constructs with the full-length PAP or 36 aa deletion

Table 10. Transformation efficiency of tobacco plants with pGA643 containing the full-length and the C-terminal serially deleted forms of the PAP gene

	No Kan	with Kan (no plasmid)[a]	pGA	pGA-(PAP)	pGA-(PAPΔ36)	pGA (PAPΔ62)	pGA-(PAPΔ131)
Shoots[b]	242	0	250	22[c]	30	164	265
Rooted/ total trans-formants[d]	0/20	-	16/20	19/22	24/30	28/30	27/30

[a] 100 μg/ml kanamycin
[b] Data from 8 plates each containing 5-8 pieces of explants.
[c] Thirty-five transformants from two other independent transformation experiments were not included. All these putative transgenic plants were analysed by Western blot and none of them contained detectable levels of PAP.
[d] More than twenty transformants were transferred to rooting medium containing 100 μg/ml kanamycin and 400 μg/ml carbenicillin.

at the C-terminus generated even fewer shoots. Most of these transformants had roots and grew into full-size plants and were used for further analysis (Table 10).

The putative transgenic plants with roots were first screened for antiviral activity using a TMV local lesion assay. Three leaves from each plant were inoculated and the number of lesions were counted. In order to obtain a relatively accurate result from the control (pGA), five cell lines transformed with the same pGA construct were infected and the numbers of local lesions were pooled and averaged. Results showed that the control produced on average 124 local lesions (from 3 leaves), while other constructs produced a varying range of lesions. All lines producing fewer lesions than the control (124) were further examined by Western blot and by large-scale antiviral activity assay.

(C) Expression of PAP in transgenic tobacco plants

Putative transgenic plants which produced fewer local lesions were tested for PAP expression by Western blot analysis, using antibodies specific to the PAP protein. Immunoblotting of protein extracts isolated from several leaves of each transgenic plant revealed the presence of a protein band that cross-reacted with the PAP-specific antibodies (Fig.33).

1 2 3 4 5 6 7

Fig.33. Western blot analysis of PAP extracted from transgenic tobacco plants transformed with construct pGA-PAPΔ36. Total proteins extracted from tobacco (200 μg) were loaded and subjected to electrophoresis. After transfer to nitrocellulose membranes, the proteins were incubated with PAP-specific antibodies and goat anti-rabbit antibodies conjugated with alkaline phosphatase. Lane 1, Commercial PAP from pokeweed plants; lane 2, pGA643 (devoid of PAP gene); lane 3, pGA-PAPΔ36c; lane 4, pGA-PAPΔ36a; lane 5, pGA-PAPΔ36b; lane 6, pGA-PAPΔ36d; lane 7, pGA-PAPΔ36e.

This protein corresponds in size to natural PAP used as a positive control (Fig.33, lane 1). No protein band could be detected from extracts of non-transgenic plants used as negative controls (lane 2). Of the four different PAP gene constructs tested, only five lines from the construct pGA-PAPΔ36 produced detectable levels of PAP (Fig.33, lanes 3-7). No visible bands were observed in the other putative transgenic lines (data not shown). This could be the result if either the expression level of PAP is below the Western blot detection limit or if a nonsense mutation of the PAP gene has occurred.

(D) Antiviral activity assay of transgenic tobacco plants

To determine the level of resistance of these transgenic plants (particularly those which produced PAP) to infection by TMV, 8-10 plants of each transgenic line from the four constructs were infected by mechanical inoculation with increasing concentrations (3 and 9 μg/ml) of TMV. Two types of controls were used: 1) non-transformed tobacco plants 2) tobacco plants transformed with pGA643 without the PAP gene. The local lesions were counted and analysed 5 days after inoculation. Results showed that all cell lines which contained detectable levels of PAP by Western blot (construct pGA-PAPΔ36) conferred resistance to viral infection (Fig.34, Table 11). Several tobacco lines which did not express detectable levels of PAP did not confer resistance to TMV infection in transgenic plants. Although PAP was not detected in construct pGA-PAPΔ62, three cell lines were resistant to viral infection (60-80%) when 3 μg/ml TMV was applied. No tobacco lines transformed with the full-length PAP gene were resistant to viral infection (Table 11).

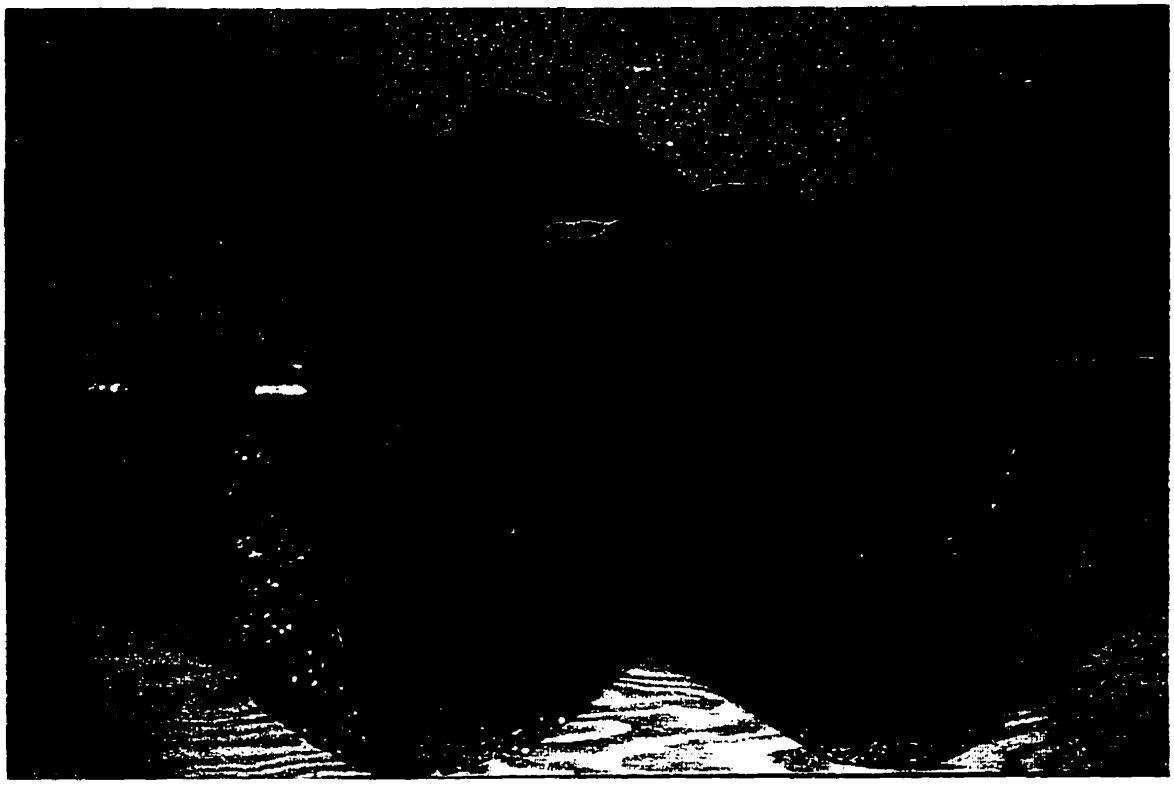


Fig.34. Anti-tobacco mosaic virus activity assay of tobacco plants. Plants transformed with binary vector pGA devoid of the PAP gene (left) and with pGA-PAPΔ36 which contains the C-terminal deleted (36 aa) PAP gene (right)
Note: very few local lesions on the right leaves.

Table 11. Anti-TMV activity assay of transgenic tobacco plants

Vector/line	(+/-)[a]	Phenotype	3 μg/ml TMV		9 μg/ml TMV	
			Exp.[b]		Exp.[c]	
			No. of lesions/ plant	Viral inhibition (%)	No. of lesions/ plant	Viral inhibition (%)
Wild type (untransformed)	-	Normal	396±87	0	346±50	0
pGA	-	Normal	383±79[d]	0	325±41[d]	0
pGA-PAP						
a	-	Normal	332±52	13	301±43	7
b	-	Normal	346±71	10	332±62	0
c	-	Normal	352±76	8	ND	ND
d	-	Normal	357±50	7	ND	ND
e	-	Normal	380±71	1	ND	ND
f	-	Normal	388±79	0	ND	ND
g	-	Normal	413±68	0	ND	ND
h	-	Normal	434±89	0	ND	ND
pGA-PAPΔ36						
a	+	Stunted	4±3	99	6±5	98
b	+	Stunted	58±12	85	52±7	84
c	+	Stunted	58±13	85	47±12	86
d	+	Normal	70±13	82	41±6	87
e	+	Normal	68±14	82	61±10	81
pGA-PAPΔ62						
a	-	Normal	78±12	80	57±13	82
b	-	Normal	139±27	64	158±28	49
c	-	Normal	154±17	60	189±32	42
pGA-PAPΔ131						
a	-	Normal	365±53	5	328±82	0
b	-	Normal	371±60	3	ND	ND
c	-	Normal	384±340	0	ND	ND
d	-	Normal	386±390	0	ND	ND
e	-	Normal	420±630	0	ND	ND

[a] The presence or absence of detectable PAP was determined by Western blot.

[b] & [c] Eight to 10 plants from each transgenic line were challenged with TMV and three leaves of each infected plant were used for the local lesion assay. [b] and [c] are two independent experiments. Standard deviation is shown.

[d] The viral inhibition was considered to be zero if any lesion number was more than the control (pGA without the PAP gene). ND, Not determined.

V Testing for Resistance of PAP-Transformed CD_4^+ Cells to HIV-1 Infection

(A) Toxicity of PAP outside cells (Done by M. Ahmed)

Before constructing a retroviral vector containing the *PAP* gene, it was important to test whether the *E. coli*-expressed PAP had a toxic effect on mammalian cells. The PA317 cell line (derived from murine fibroblast NIH 3T3 cell line) was treated with different concentrations of PAP. A PAP concentration of less than 0.05μg/ml added to the culture medium had a low toxicity on these cells and a concentration of 5 μg/ml gave only 25% cell growth inhibition (Table 12). However, a concentration of 5 μg/ml PAP combined with retrovirus particles killed all of the cells, indicating virus infected cells are more sensitive to PAP (Table 13).

Table 12. Extracellular toxicity assay of *E. coli*-expressed PAP on mammalian cells

PAP concentration (μg/ml)	Confluence level[a] (%)	Toxicity level[b] (%)
5.000	75	25
0.500	80	20
0.050	95	5
0.005	100	0
0.000 (control)	100	0

PA317 cells derived from murine fibroblast NIH 3T3 cell line (3×10^5) were incubated with different concentrations of PAP. The confluence of cell colonies were recorded on the third day.
[a] The ratio of surface area covered by monolayer cells relative to the total surface area.
[b] Toxicity is described as 100% (control) - confluence level of the sample.

Table 13. Antiviral activity assay of *E. coli*-expressed PAP on mammalian retroviral vectors

PAP concentration (μg/ml)	No. of colonies	Viable cell count	Inhibition of infectivity (%)[a]
5.000	0	0	100
0.500	2197	1.14×10^6	40
0.050	3262	1.62×10^6	10
0.005	3123	5.34×10^6	13
Positive control (Retrovirus alone no PAP)[b]	3609	4.38×10^6	0
Negative control (No retrovirus, no PAP)[c]	0	0	

PA317 cells (50% confluence) were incubated with 100 μl of recombinant retroviral particles (derived from murine leukaemia virus) and different concentrations of PAP. Since the viral particles contained *neo* gene for G418 resistance, only the infected cells survived in the presence of G418, while the un-infected cells died and were washed away.

[a] Inhibition percentage is based on number of colonies.
[b] Highest number of colonies due to the lack of PAP effect.
[c] No cell growth under G418 selection due to the lack of viral particles.

(B) Construction of the N-terminal signal deleted PAP gene ($PAP_{\Delta N}$) into retroviral vectors

The PAP gene ($PAP_{\Delta N}$) was introduced into retroviral vectors to be expressed in $CD4^+$ cells. To insert the $PAP_{\Delta N}$ gene into two *Sfi*I sites of the retroviral vector, two oligonucleotide

primers were designed according to the known cDNA sequence of the *PAP* gene. The upstream primer, $P_{HIV\Delta NF}$ (5'CCCCCGGGCC-AAGTC-GGCC-GCCACCATGGTGAATACAATCAT-CTACAAT 3' starts from the mature protein sequence with the addition of an *Sfi*I recognition sequence, the Kozak consensus sequence for the ribosome-binding site (5'GCCGCCACC3') and an ATG start codon (underlined), while the downstream primer, $P_{HIV\Delta NR}$ 5' TTCCCCCGGCCAAGTCGGCCTTATGATCAGAATCCTTCAAATAGA 3' starts 5 bases after the stop codon at the opposite strand with an additional *Sfi*I recognition sequence. A PAP fragment (about 900 bp) without the N-terminal signal sequence was obtained by PCR. The sequence of this fragment was verified by restriction endonuclease analysis (*Acc*I, *Bst*XI or *Sac*I). The PCR product was digested with *Sfi*I and then ligated into the retroviral vectors pMoTN-Ti-Ri at the *Sfi*I sites. After bacterial transformation one of the colonies pUCMoTN-Ti-PAP-Ri-Ter (for short pA1) was found to contain the $PAP_{\Delta N}$ gene in sense orientation (Fig.35).

A synthetic DNA duplex [two oligonucleotides, $P_{HIV\Delta PAPF}$ (5'ATCGATCCGGATCCTCG-CGAGCGGCCGCACT3') and $P_{HIV\Delta PAPR}$ (5'GCGGCCGCTCGCGAGGATCCGGATCGAT-ACT3'], which contained the following restriction sites *Cla*I, *Acc*III (*Bsp*pEI), *Bam*HI, *Nru*I, *Not*I in place of the PAP gene, was cloned and used as a negative control (pA2).

(C) Detection of integrated $PAP_{\Delta N}$ gene in transformed MT4 cells

The retroviral vectors were transfected into the Psi-2 packaging cell line first, and then into PA317 packaging cell line which produced vector particles having the ability to infect human CD_4^+ lymphocyte-derived MT4 cells. The presence of the $PAP_{\Delta N}$ gene in the genome of MT4 cells was confirmed by PCR analysis using oligonucleotides ($P_{HIV\Delta NF}$ and $P_{HIV\Delta NR}$) flanking both

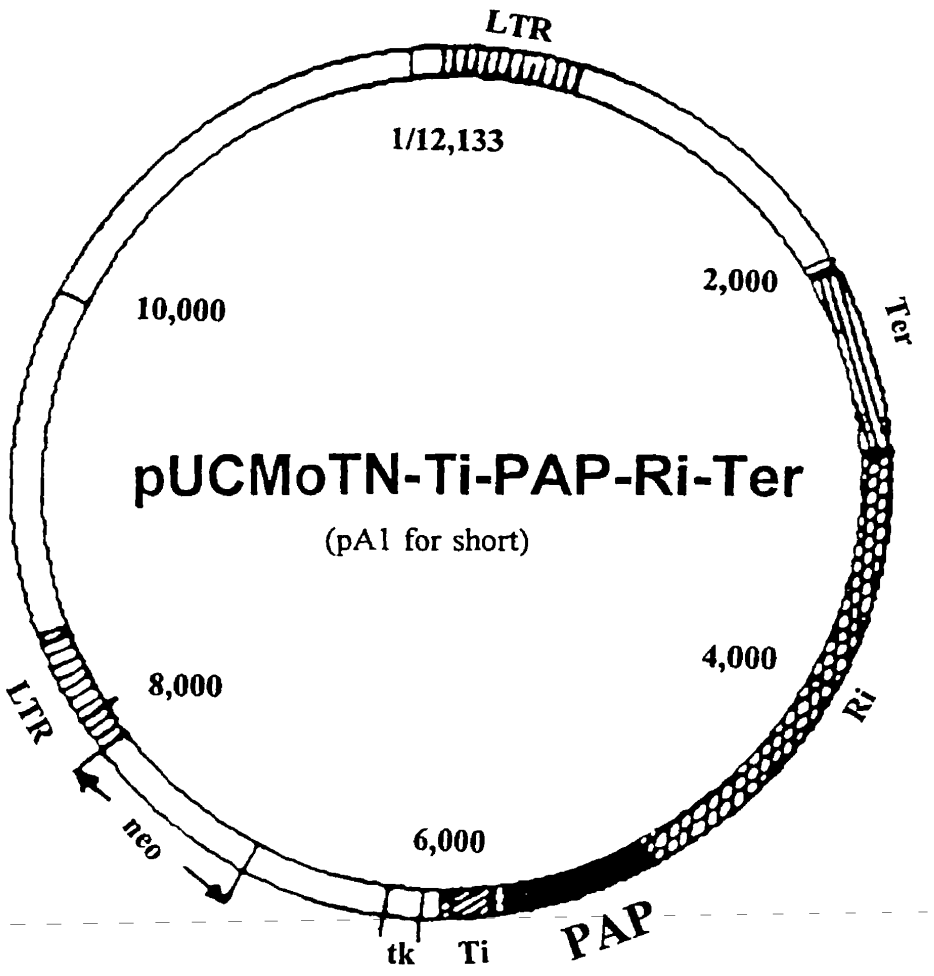


Fig.35. Construction of a murine leukaemia virus derived retroviral vector containing the pokeweed antiviral protein (PAP) gene. The N-terminal signal deleted PAP gene ($PAP_{\Delta N}$) was inserted into the above retroviral vector at *Sfi*I sites, so that the PAP gene was controlled by HIV-1 regulatory proteins, Tat and Rev. Correct orientation of the PAP gene was confirmed by digestion with several restriction enzymes. LTR, long terminal repeat; Neo, Neomycin resistant gene; Ti, Tat inducible promoter; Ri, Rev inducible sequence; Ter, Terminator; tk, thymidine kinase promoter.

sides of the $PAP_{\Delta N}$ gene. A 900 bp PCR product appeared in MT4 cells transformed with vectors containing the $PAP_{\Delta N}$ gene but not in MT4 cells transformed with vectors devoid of the $PAP_{\Delta N}$ gene (Fig.36). To detect if PAP mRNA was expressed from the Tat-inducible promoter, RT-PCR

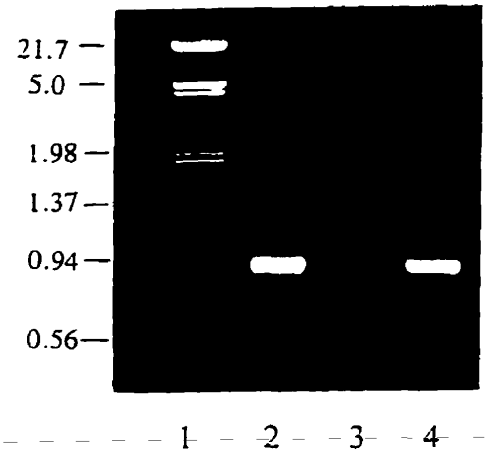


Fig.36. PCR of genomic DNA from MT4 cells transformed with retroviral vectors containing the $PAP_{\Delta N}$ gene. Lane 1, DNA size markers in kb; lane 2, plasmid pBS$^+$-PAP_{anti} as a positive template control; lane 3, genomic DNA from normal MT4 cells; lane 4, genomic DNA from MT4 cells transformed with retroviral vectors containing the $PAP_{\Delta N}$ gene. Note the presence of a 900 bp fragment in lanes 2 and 4 but absent in lane 3.

was performed using RNA isolated from MT4 cells. These cells were induced with phytohaemagglutinin (Tong-Starksen *et al.*, 1989) for the expression of PAP mRNA and total RNA was extracted for RT-PCR analysis. Reverse transcription was performed using a single primer $P_{HIV\Delta NR}$ which is complementary to the 3' flanking region of the $PAP_{\Delta N}$ gene in the retroviral vector. After phenol-chloroform extraction, this cDNA was then amplified by PCR using two oligonucleotides [$P_{HIV\Delta NF}$ (complementary to the 5' flanking region) and $P_{HIV\Delta NR}$]. The presence of a 900 bp fragment confirmed the expression of the PAP gene (Fig.37).

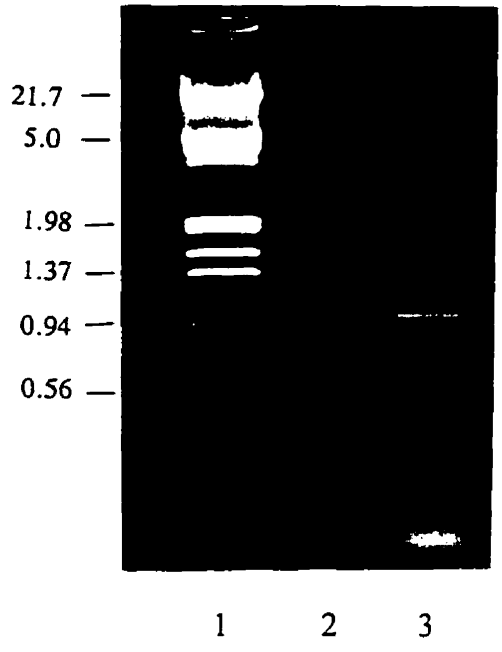


Fig.37. Reverse transcriptase PCR of total cellular RNA from MT4 cells transformed with retroviral vectors containing the *$PAP_{\Delta N}$* gene. Lane 1, DNA size markers in kb; lane 2, MT4 cells transformed with retroviral vectors devoid of the *PAP* gene; lane 3, MT4 cells transformed with retroviral vectors containing the *$PAP_{\Delta N}$* gene.

(D) Challenge with HIV-1

This experiment was conducted by Azim Ladha in Dr. Joshi's laboratory. MT4 cells transformed with the PAP gene were infected with HIV-1. Culture supernatants were collected from the cells 15 days after infection. Detection of HIV yield was performed by measurement of p24 antigen (a core antigen of HIV virion) using ELISA. The results obtained from two independent experiments showed practically no difference in HIV-1 antigens between cell lines with and without the PAP gene (Table 14).

Table 14. p24 levels determined by ELISA in culture supernatants from MT4 cell lines following HIV-1 infection

A1 ($PAP_{\Delta N}$ gene)	250
A2 (devoid of the PAP gene)	380

Data are arbitrary absorbance units derived from two independent experiments.

(E) Sequencing of the entire ORF of the $PAP_{\Delta N}$ gene in the retroviral vector pA1

Sequencing of the entire open reading frame of the $PAP_{\Delta N}$ gene in the retroviral vector showed that A^{561} was deleted, resulting in early terminated PAP (187 aa). To determine if this premature PAP had any biological activity, *in vitro* transcription and translation were performed.

(F) *In vitro* transcription and translation of the $PAP_{\Delta N}$ cassette from the retroviral vector pA1

The $PAP_{\Delta N}$ cassette containing the Ti- promoter, Kozak ribosome binding site, $PAP_{\Delta N}$ gene, Ri segment and Ter terminator in the retroviral vector was subcloned into pBS$^+$ downstream of the T7 promoter. This was achieved by digestion of pA1 with *Asp*718 (*Kpn*I) to release the cassette fragment and the fragment was inserted at *Asp*718 site in the pBS$^+$. The orientation of the insert was verified by digestion with the unique sites for *Xho*I and *Pst*I. One of the subclones was designated pSA1 and used for further study. A similar subclone (pSα1) containing the $PAP_{\Delta N}$ with a frame shift (deletion of G^{21}) was obtained for the control clones. Both subclones pSA1 and pSα1 were linearized with *Hind*III, deproteinized and transcribed *in vitro* with T7 RNA polymerase (Fig.38). The transcribed mRNA was then added into the *in vitro* rabbit reticulocyte

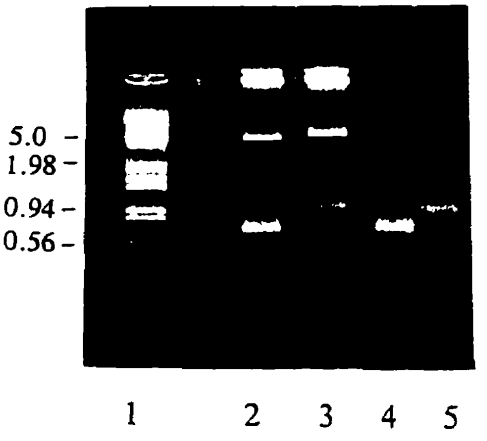


Fig.38. *In vitro* transcription of the $PAP_{\Delta N}$ gene derived from retroviral vector pA1. Two subclones (pA1 and pα1) were linearized with *Hind*III, and the PAP gene cassettes were transcribed from T7 promoter. Lane 1, DNA size markers in kb; lane 2, pA1; lane 3, pα1; lane 4, pA1 after RQ1 DNase treatment; lane 5, pα1 after RQ1 DNase treatment.

translation system. Results obtained showed that pSA1 transcripts partially inhibited the *in vitro* translation system (Fig.39, lane 2) while those of pSα1 seem to have no noticeable effect (Fig.39, lane 3), indicating that premature PAP may have some ribosome-inactivating activity.

(G) Testing of the inducible killing of cells expressing Tat and Rev (Done by Azim Ladha)

To test if $PAP_{\Delta N}$ in the retroviral vectors was induced by Tat and Rev proteins and if the expressed PAP killed the infected cells, cells (cell line GP+E) expressing both Tat and Rev were infected by vectors containing the PAP gene or not, which were released from the packaging cell line Psi-2. It was found that no difference in cell confluence was observed after G418 (an antibiotic similar to kanamycin) selection (data not shown).

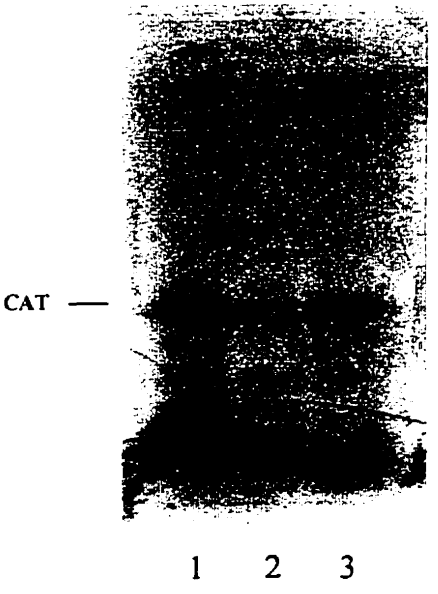


Fig.39. *In vitro* translation inhibition assay of the $PAP_{\Delta N}$ gene derived from retroviral vector pA1. Same amounts (1 μg) of *in vitro* transcribed RNA from two subclones were added into the *in vitro* translation system. Lane 1, CAT mRNA was used as a template (positive control); lane 2, RNA transcripts from pSA1; lane 3, RNA transcripts from pSα1. Note the CAT protein band in lane 2 is very weak.

DISCUSSION

Isolation and Constitutive Expression of the Full-length PAP Gene in *E. coli*

Pokeweed antiviral protein (PAP or PAP I) from *Phytolacca americana* is a highly specific N-glycosidase. This enzyme inhibits protein synthesis by removing a single adenine from intact ribosomes (A^{4324} in 28S rRNA and A^{2660} in 23S rRNA) of both eukaryotes and prokaryotes (Irvin and Uckun, 1992; Barbieri *et al.*, 1993). Although PAP has long been known to be antiviral, its detailed study, particularly at the molecular level, is a recent event. In this study, the PAP cDNA fragment (1.1 kb) encoding the entire PAP [313 amino acids (aa)], including the N-terminal leader signal (22 aa), the mature PAP (262 aa) and the C-terminal extra-peptide (29 aa), as well as a 3' untranslated region, was successfully isolated from pokeweed leaves and cloned in plasmid *p*BS$^+$. The entire open reading frame of the PAP gene was sequenced (Fig.16) and found to be identical to that previously reported (Chen *et al.*, 1991).

In order to study the PAP gene, attempts were made to express the full-length PAP gene (PAP_f) in *E. coli* cells. It is generally accepted that genes coding for toxic proteins should be expressed under the control of an inducible promoter. Such genes are not transcribed in the absence of an inducer and transcription is activated by the inducer. For this reason, only inducible promoters have been employed thus far for the expression of PAP and other ribosome-inactivating protein (RIP) genes in bacteria (Chen *et al.*, 1993; Chaddock *et al.*, 1994 and Barbieri *et al.*, 1993). Although Chen *et al.* (1993) expressed the PAP_f gene under an inducible *lac* promoter and obtained low yields (0.37 to 0.9 mg/l bacterial culture) which was explained by PAP toxicity to the host cells, the mechanisms by which *E. coli* cells express PAP and resist the toxic effect of

the expressed PAP remain unknown. It is possible that the accumulation of PAP in inclusion bodies serves to protect *E. coli* ribosomes. Further, the presence of the N-terminal leader sequence could also be used to anchor the PAP to the *E. coli* cell wall as is the case in pokeweed plants.

To test if the low yield of PAP under the control of the inducible *lac* promoter is due to its toxicity, the expression of the same gene under a strong constitutive promoter was examined and the results compared to those obtained by the inducible *lac* promoter. Both the constitutive vector (pP_1R_9) and the inducible vector ($pP_1O_1R_9$) have been successfully used for high-level expression of various eukaryotic genes in *E. coli* (Jay *et al.*, 1984, 1991; Ivanov *et al.*, 1987a, 1987b and 1990).

Surprisingly, it was found that the level of the full-length protein (PAP_f) was 6-10 times higher using the constitutive plasmid (pP_1R_9) than that using the inducible vector ($pP_1O_1R_9$) (Table 3). This result contradicted the assumption that the low PAP_f yield was due to its toxicity to the host cells (Chen *et al.*, 1993). In order to investigate the mechanism for the different yields obtained with the two vectors, the level (Table 3) and stability (half-life, data not shown) of PAP_f mRNA was also studied. No significant difference in PAP mRNA stability between these two vectors was observed, although the PAP mRNA yield from the inducible vector was slightly lower. If the mRNA yield was taken into account and the protein yield was normalized to the content of mRNA, the PAP_f mRNA transcribed from the inducible plasmid $pP_1O_1R_9$-PAP_f was translated 4-5 times less efficiently than PAP mRNA obtained with the constitutive (pP_1R_9-PAP_f) plasmid. Thus, it seems that the translation but not the transcription is the main factor for the low yield of PAP when using the inducible vector.

To determine why PAP_f mRNA containing a *lac* operator sequence (inducible vector) at the 5'-terminus was translated with a lower efficiency compared to that devoid of such a sequence (constitutive vector), 5'-terminal regions of PAP_f mRNAs (50, 100 and 200 nt) transcribed from pP_1R_9-PAP_f, $pP_1O_1R_9$-PAP_f, as well as from the commercial inducible plasmid *p*KK233-2, were folded using the computer program DNASIS. Results for the three different sized 5' regions showed that the Shine-Dalgarno sequence was involved in a stable stem structure when mRNAs contained a *lac* operator sequence but was in a looser secondary structure when mRNAs were devoid of this sequence (Fig.18A). Secondary structure of mRNA has been demonstrated to affect translation initiation (De Smit and van Duin, 1990; Ganoza and Louis, 1994). Based on the results from this study (Table 3 and Fig.18), the lower protein yield obtained using an inducible (*lac*) promoter could be the result of mainly less efficient initiation of translation in comparison to that with the constitutive promoter (P_1R_9). The low yield of mRNA in the *E. coli* cells transformed with the inducible vector was probably due to the negative feedback effect of the inefficient translation on the transcription efficiency (Alexciev *et al.*, 1989; Gigova *et al.*, 1989; Ivanov *et al.*, 1992; Kurland, 1992).

Biological (antiviral and ribosome-inactivating) activity of the full-length PAP protein (PAP_f) expressed in *E. coli* was tested by an anti-tobacco mosaic virus local lesion assay and by an *in vitro* rabbit reticulocyte translation system. Results (Table 5 and Fig.21) from both assays showed that PAP_f had a similar inhibitory effect as the natural (mature) PAP. These data reveal that in spite of the presence of the N-terminal signal peptide, the recombinant PAP_f protein is active and its activity is comparable with that of the mature PAP protein.

Immunogold ultrastructural studies demonstrated that the recombinant PAP_f protein was

localized inside the cells in the form of inclusion bodies, although some gold grains were found close to the periplasmic space (Fig.19). Western dot-blot analyses showed that the concentration of PAP_f in the culture medium did not exceed 2% of the total protein and no more than 5 to 10% of the PAP_f was found in the cell periplasmic space (after mild osmotic lysis). Both protein analysis and ultrastructural data indicate that the recombinant PAP_f protein has a primarily intracellular cytoplasmic localization.

The results presented above raise the following question: How can *E. coli* constitutively produce and tolerate a protein which should be active against its ribosomes? It might be explained by assuming that either *E. coli* ribosomes are resistant since some cereal ribosomes were reported to be resistant to their own RIPs (Leah *et al.*, 1991; Walsh *et al.*, 1991) or that the full-length PAP is inactive *in vivo* and it is activated after disruption of recombinant cells. It appears the former is unlikely because an *in vitro* assay found that *E. coli* ribosomes were inactivated by PAP (Habuka *et al.*, 1991b). Rather, it is more likely that the full-length PAP is inactive *in vivo* but activated after disruption of recombinant cells. The fastest way to inactivate proteins is by precipitation or export out of the cell. The finding that the recombinant full-length PAP was found mainly in the form of inclusion bodies in the cytoplasm supports the proposal that PAP might aggregate by itself or by binding to some host specific proteins such as chaperon proteins that help in folding and binding. It is assumed that precipitation to form inclusion bodies and export out of the cell may be an efficient way of inactivating PAP, otherwise, spontaneous mutants of the full-length PAP gene would have been recovered. It seems possible that this mechanism of inclusion body inactivation is inapplicable for PAP proteins devoid of the N-terminal signal peptide, since wild-type N-terminal signal peptide deleted PAP is lethal to *E. coli*

cells (see below).

It was observed by Chen *et al.* (1993) that the PAP_f protein expressed by an inducible promoter in *E. coli* had two forms, one shorter (29 kDa) corresponding to the size of the mature PAP protein. In this study, two forms in approximately equimolar amounts were found in *E. coli* cells expressing the PAP_f gene under a constitutive promoter. A similar pattern (two forms but with different molecular masses compared to those of the full-length PAP), was also observed in the protein recovered from the cell culture medium (Fig.20, lane 2). This result implies the PAP from the spontaneously lysed cells rather than from the secreted expression since the extrapeptides at both N- and C-termini of the secreted PAP should be cleaved and the PAP should have a single band. If the distribution of the processed PAP within the cells (periplasm and cytoplasm) is analysed, approximately 50% of total PAP is truncated (see Fig.20, lane 3) and only about 10% is in the cell periplasm, indicating that approximately 40% of truncated PAP is localized in the cytoplasm. However, considering the extremely high toxicity of PAP devoid of the N-terminal signal peptide to *E. coli* cells (Table 6, discussed later), it seems unlikely that the truncated PAP resulted from the N-terminal deletion. Most likely the truncated protein is a product cleaved at its C-terminus and it becomes less toxic (Kim and Robertus, 1992).

The results described above demonstrate that the PAP_f gene can be constitutively expressed and the expression level is higher than that obtained with an inducible (*lac*) vector containing the same promoter and SD sequence. *E. coli* cells can tolerate and accumulate a substantial amount of PAP_f in the cytoplasm. The PAP_f in the cytoplasm is likely inactivated by aggregation or improper folding and becomes active when isolated and renatured. The lower protein yield obtained with the inducible *lac* promoter is perhaps due to an inefficient initiation

of translation of PAP_f mRNA containing the *lac* operator sequence rather than protein toxicity. Therefore, this constitutive expression system may be very helpful in expressing recombinant PAP and possibly other ribosome-inactivating proteins.

Effect of Mutations on the Expression in *E. coli* and Biological Activities of PAP

Although the *PAP_f* gene has been isolated and its three-dimensional structure refined to 2.5 Å resolution, the function of both N- and C-terminal regions of PAP and other type I RIPs is poorly characterized. Instead, much work has focused on the ricin A-chain due to its lack of toxicity to *E. coli* cells (Munishkin and Wool, 1995; Simpson *et al.*, 1995). As described before, the full-length PAP_f consists of two extra peptides one located at the N-terminus (22 aa) and the other at the C-terminus (29 aa) in addition to the mature protein (262 aa). Here the effects of both N- and C-termini were studied by deletion of the N-terminal signal (22 aa) and deletion of the C-terminal regions (62 aa) using the constitutive expression vector. Since it is difficult to distinguish toxic from non-toxic forms of the PAP genes if an inducible promoter is used, a strong constitutive promoter was chosen to express the engineered PAP gene constructs. Thus, a large amount of (even of a moderate toxicity) protein will be produced and would be expected to kill the cells and cell mortality can be used as a phenotype for protein toxicity.

Cloning experiments with the $PAP_{\Delta N}22$ (22 codons of the N-terminal signal peptide removed) construct confirmed the results of Chen *et al.* (1993). *E. coli* colonies containing the $PAP_{\Delta N}22$ gene were isolated only if the construct was mutated or in the reverse orientation. Similar results were obtained with the block-deleted genes $PAP_{\Delta N}49$, $PAP_{\Delta N}71$ and $PAP_{\Delta N}89$ as well as with the 5' to 3' progressive deletion mutants $PAP_{\Delta N}\leq 107$ (Meng, A., 1997). As a result,

it was impossible to find any clone containing a wild-type PAP gene in the sense orientation until 123 or more codons were removed from the N-terminal coding region, i.e., all sequenced clones ($PAP_{\Delta N} \leq 107$) contained only a mutant form of the PAP gene, non-mutated PAP genes were found only among the clones from the $PAP_{\Delta N} > 123$ series (Meng, A., 1997). These results indicate that PAP mutants with deletions up to 107 aa are toxic. Since it is generally believed that toxicity is due to the ribosome-inactivating activity. Thus, the first 107 aa may not be involved in the active site of PAP.

This result is not consistent with the commonly accepted hypothesis that Tyr94 is involved in the active site. Sequence comparison in the RIP family of toxins revealed that Tyr94, Lys145, Glu198 and Arg201 (in cDNA) are invariant (Monzingo *et al.*, 1993). These conserved residues have been extensively studied in ricin and models for the active site were proposed mainly on the basis of mutagenesis studies (Frankel *et al.*, 1990). Mutation study found that substitution of Tyr94 in the ricin A chain decreased the activity, suggesting Tyr94 may be involved in the active site (Frankel *et al.* 1990). However, results from this study with the PAP gene suggest that the Tyr94 residue is not an integral part of the putative active site. It is possible that Tyr94 in PAP may be close to the active site but may not be an integral part of the active site so that mutation or deletion decreases but does not abolish the activity. This suggestion is supported by Ke *et al.* (1997) who reported that removal of 100 aa from the N-terminus of trichosanthin (another single chain ribosome-inactivating protein) still retained biological activity (Ke *et al.*, 1997).

Since deletion of 107 codons did not abolish the PAP activity but deletion of a further 16 codons (total 123 aa) did abolish the activity (Meng, A., 1996), it appears that the residues between 107 and 123 may be involved in the active site.

The mechanism for the high level expression of the full-length PAP gene but not the N-terminal signal deleted PAP gene remains to be understood. Chen *et al.* (1993) postulated that the N-terminal signal peptide was recognized by *E. coli*, used for secretion and was cleaved after the protein was exported out of the cell. It is also possible that the N-terminal extrapeptide may be required to form inclusion bodies for the temporally inactivation of PAP. The N-terminal extrapeptide may help by binding PAP themselves or by binding other cellular proteins such as chaperons involved in aggregation. Therefore, the *E. coli* cells tolerated the expression of the full-length PAP in the cytoplasm but did not tolerate the expression of the N-terminal extrapeptide-deleted PAP gene.

In this study, an extremely high mutation rate of the PAP gene was observed. High mutation rates were also described by Dore *et al.* (1994) but the mutagenic source was not determined. It could be hypothesized that the PCR conditions themselves were mutagenic. However, the results from DNA sequence analysis of PAP constructs inserted in reverse orientation into the same expression vector (no clones with altered nucleotide sequence were found) disagree with such an assumption. It seems very likely that the mutagenesis of the PAP genes is an endogenous rather than exogenous process used as a protective mechanism against the toxicity of the gene products (Chaddock *et al.*, 1994).

The role of the C-terminal region of the PAP gene was also investigated. The PAP gene contains a C-terminal extra peptide (29 aa) in addition to the mature PAP (262 aa) and the N-terminal peptide (22 aa). Results from other RIP studies (saporin and barley RIP) revealed that the C-terminal extra peptides were necessary for transport into vacuoles (Bednarek *et al.* 1990). However, the C-terminal extra peptide in ricin may also act as an inactivator to prevent self-

inactivation of host ribosomes (Butterworth and Lord, 1983). Since the full-length PAP (including the sequence for the C-terminal extra peptide) expressed in *E. coli* was biologically active, it seems that the C-terminal extra peptide of PAP did not act as an inactivator.

Based on amino acid identity comparisons among the RIPs, the active site of the mature PAP was proposed to be in the amino-terminal and middle region (Irvin & Uckun 1992). However, direct evidence for the function of the C-terminal region (more than the C-terminal extra peptide) is not available. Funatsu *et al.* (1991) compared the amino acid sequences of 11 RIPs and found the 40 amino acid residues at the C-terminus were not well conserved, suggesting these variant regions were not involved in the catalytic function of RIPs. A series of protein swap experiments with PAP and the ricin A-chain showed that the C-terminal domain in PAP does not contribute significantly to ribosome recognition. Results from progressive mutagenesis demonstrated that deletion up to 48 aa from the C-terminus (including the 29 aa extra peptide) did not affect the biological activity (Meng, A., 1996), confirming that the C-terminus (up to 48 aa) is not involved in the active site. However, a deletion of 62 amino acids from the C-terminus (33 aa plus 29 aa extra peptide) dramatically decreased the antiviral activity (from 93% to 28%). An *in vitro* protein synthesis inhibition assay showed that the same C-terminal truncated PAP protein retained approximately 10% ribosome-inactivating activity compared to that of the full-length PAP (Fig.25). The fact that deletions of 48 and 55 amino acids from the C-terminus (including the extra peptide) resulted in no activity reduction, or a reduction by only 8% respectively, but deletion of 62 aa decreased activity by 65% suggests the eight amino acids from 55 to 62 may play an important role in retaining proper protein conformation and therefore, antiviral activity.

Parallel reduction of both ribosome-inactivating activity and antiviral activity in the PAP mutant $pPAP_{\Delta C}62$ suggests that antiviral activity resulted from the ribosome-inactivating activity. Further studies by progressive deletion from the 3' end of the PAP gene support this hypothesis (Meng A., 1996). However, this work is not consistent with the results obtained by Tumer *et al.*(1997). They reported that the antiviral activity of PAP is separated from its ribosome-inactivating activity. In other words, transgenic tobacco plants containing a C-terminal deletion mutant (54 aa from the cDNA) of PAP inhibited viral infection but did not depurinate host (tobacco plants) rRNA. Thus, it appears that most of the C-terminus may be required for the toxicity and depurination of tobacco rRNA *in vivo*. The reason for the discrepancy is possibly due to different assay systems. For instance, both expression level and ribosome-inactivating activity of the truncated PAP (62 aa deletion) were low. In an *E. coli* system, both the expression level and ribosome-inactivating activity of the truncated PAP was only approximately 10% compared to the full-length PAP, respectively. Thus, the low activity may not be easily detected. Moreover, it is well known that mammalian ribosomes are most sensitive to PAP, plant ribosomes are second, and bacterial ribosomes the least sensitive. In plants, ribosomes from different species also show various sensitivities (Irvin and Uckun, 1992). In our laboratory, either rabbit red blood cell lysate or wheat germ lysate were used and both were found to be very sensitive to PAP, while the ribosomes from tobacco may not be as sensitive as those from wheat germ or blood cells. It is likely that the C-terminal truncated PAP may have trace ribosome-inactivating activity which inactivated ribosomes from blood cells and wheat germ cells (in this study) but which failed to inactivate ribosomes from tobacco plants (Tumer *et al.*, 1997). The failure to inactivate ribosomes from tobacco plants could be caused by less sensitivity of tobacco ribosomes to PAP

in nature or the interference of impurities (obtained during the isolation of tobacco ribosomes) in the *in vitro* translation assay system. Thus, the truncated PAP may be still active but the activity may be very low.

Characterization of Novel Translational initiators for High Expression of PAP

Due to its great potential as an antiviral and anticancer agent (Aron and Irvin, 1980; Chen *et al.*,1991; Myers *et al.*, 1991; Jansen *et al.*, 1993), mass production of PAP by recombinant DNA technology would be very useful and more efficient translational initiators are therefore desirable to produce higher levels of expression. Moreover, studies of non-SD translational initiators may answer the basic biological question of how mRNA and ribosomes interact during translation.

Originally designed as a negative control (no SD sequence), the PL (ACCUACUCGAGUUAG) sequence (a polylinker from plasmid pP_I-PL) placed in front of the initiation codon was capable of initiating translation of PAP_f mRNA in *E. coli* cells. Since this sequence was not homologous to the SD sequence it could not be involved in the canonical SD-anti-SD interaction. Either box I or box II in PL was capable of initiating translation but both were required for maximal PAP gene expression. The most unexpected result was that the PL sequence was twice as efficient as the SD consensus sequence (Table 9). These results indicate that the most probable sites for interaction of 16S rRNA with the PAP_f mRNA carrying the PL sequence are the nucleotide domains 1434-1440 and 507-513 on the 16S rRNA. The question arises, however, as to whether these two distinct domains are in close proximity on the surface of the 30S ribosomal subunit, in order to act as a non-SD translation initiator.

Nucloetides 507-513 and 1434-1440 are located on two ribosome domains "530" and "1400" regions or neighborhoods based on nucleotides G^{530} and U^{1440} in the 16S rRNA. In spite of extensive work the localization of the two domains are still controversial. It is well established that the nucleotides from these two neighborhoods lie in the mRNA track (McCarthy & Brimacombe, 1994) and are involved in the organization of the ribosome decoding centre (Malhotra & Harvey, 1994; Brimacombe, 1995; Easterwood & Harvey, 1995; Frank *et al.*, 1995; Noller *et al.*, 1995; Oakes & Lake, 1990; O'Connor *et al.*, 1995, and Stern & Purohit, 1995). Numerous studies show that nucleotides 1399-1409 and 1492-1504 belong to the A (acceptor) and P (peptidyl) sites of the *E. coli* ribosome (Brimacombe, 1995; Heilek & Noller, 1996; Moazed & Noller, 1990 and Döring *et al.*, 1994). The results described in Fig.27 show that nucleotide domain 1434-1440 (corresponding to box I in PAP_f mRNA) is localized in a bulge-stem area outside the A and P sites (i.e. between nucleotides 1409 and 1492).

Whereas the assignment of the "1400" neighborhood nucleotides to the ribosomal A and P sites is generally accepted, disagreement about the positioning of the 530 neighborhood persisted until recently. Based on cross-linking data, Brimacombe and co-workers (Dontsova *et al.*, 1992 and Rinke-Appel *et al.*, 1993) concluded that the two "530" and "1400" neighborhoods are organized in close proximity on the surface of the 30S ribosomal subunit. This assumption contradicted the protein foot-printing analysis by Noller and co-workers (Powers and Noller, 1994) who believed that the two neighborhoods were widely separated. However, more recent studies using a hydroxyl radical foot-printing approach (Heilek and Noller, 1996) showed that the cys-labelled S5 protein cleaved 16S rRNA at positions 510, 530, 926 and 1400 and found that the distance between the different cleavage sites was 40 Å. Since the evidence from the hydroxyl

radical approach is direct while that from the conventional foot-printing is indirect, it is more likely that the close proximity of the two neighborhoods on the 30S ribosomal subunit.

Although the function of the "530" neighborhood is still speculative it has been demonstrated that nucleotides 505-507 and 524-526 are involved in a pseudoknot structure capable of changing the conformation of the 530 loop (Moazed & Noller, 1990). Two of the three pseudoknot nucleotides overlap the nucleotide domain 507-513, which is complementary to box II in PAP mRNA (Fig.26B). The results presented in this study show that the entire PL sequence is more efficient in initiating translation than box I or box II alone. The logical explanation for this result is that the PL sequence (containing both boxes) interacts simultaneously with both nucleotide domains 507-513 and 1434-1440. Thus, results of this study provide independent evidence supporting the concept that the two "530" and "1400" neighborhoods are situated in close proximity (probably on the same side) on the surface of the 30S ribosomal subunit. From the efficient initiation of translation by the PL sequence it was further concluded that nucleotides belonging to the two neighborhoods in 16S rRNA were also involved in the formation of an alternative mRNA binding site which was twice as efficient in binding PAP mRNA as the conventional anti-SD binding site.

How is the new mRNA binding site organized in the 30S ribosomal subunit? Although crosslinking or other structural evidence for the location of the "anti-PL" binding site on the three-dimensional model of 30S ribosomal subunit was not addressed in this thesis, the "anti-PL" binding site can be positioned opposite the anti-SD binding site on the side of the cleft at the lower part of the head of 30S ribosomal subunit. This positioning was based on the following facts and concepts: (1) The proximity of "530" and "1400" neighborhoods as confirmed by others

(Brimacombe, 1995; Döring *et al.*, 1994 and Heilek & Noller 1996); (2) the localization of ribosomal proteins which are known to cross-link nucleotides from both neighborhoods (Frank *et al.*, 1995; Rinke-Appel *et al.*, 1994); (3) mRNA-rRNA cross-linking data (Brimacombe, 1995); (4) Recent concepts regarding the three-dimensional organization of the functional sites on the *E. coli* ribosome (Brimacombe, 1995; Easterwood & Harvey, 1995; Frank *et al.*, 1995 and Malhotra & Harvey, 1994); (5) The requisite distance between the mRNA binding site and the P site (based mainly on studies with SD containing mRNAs) (Dontsova *et al.*, 1991).

The results presented in this study raise several questions: Why is the PL sequence stronger as a translational initiator than the SD consensus sequence? Why is box II in PL more efficient than box I? What is the mechanism for the leaky translation after mutating both box I and box II?

One reason for the higher translational efficiency of PL in comparison to the conventional SD sequence could be the stronger binding of mRNA to the ribosome, i.e., the lower free energy (ΔG) of interaction with 16S rRNA. The predicted free energy for each of the two boxes (in PL) is $\Delta G = -10.8$ Kcal/mol. Ideally, the total energy of interaction of PL with the 16S rRNA should be $\Delta G = -21.6$ Kcal/mol, which is much lower than that of the SD consensus sequence ($\Delta G = -12.8$ Kcal/mol). In practice however, this seems impossible for at least three reasons: (1) the sequence in the 16S rRNA interacting with PL is interrupted; (2) some of the nucleotides belonging to the domain 1434-1440 (complementary to box I) may be involved in a double-stranded structure (see Fig.27) which could also explain the lower efficiency of box I in initiating translation in comparison with box II; (3) it is very likely that some of the nucleotides forming the PL binding site on the 30S subunit are involved in RNA-protein interactions (unlike the anti-SD binding site). Although the real interaction energy between mRNA and rRNA is not available,

it is possible that the interaction of the PL sequence with 16S rRNA is stronger than that of the SD sequence.

The higher efficiency of PL in comparison with the SD sequence may be explained by the effect of the two downstream boxes (box III and box IV) found in the coding region of the PAP_f gene. One of the boxes (box III) is ten nucleotides long and starts with the initiation codon while the other (box IV) is eight nucleotides long and is found 43 nt downstream of the initiation codon. Both boxes showed quite a low energy of interaction (Fig.29) with the corresponding domains 1320-1329 and 541-548 in 16S rRNA one of which belongs to the "530" neighborhood. More recent studies have shown that efficient gene expression in *E. coli* depends on both the nucleotides upstream as well as downstream from the AUG nucleotide sequences when they are complementary to 16S rRNA. In most cases, the "downstream box" is found among the first 15-26 nt from the initiation codon and it is complementary to nucleotides 1469-1483 in 16S rRNA (Sprengart *et al.*, 1996). Box III in the PAP gene does not match the "anti-downstream box" sequence. However, neighbour nucleotides (around A^{1360}) to the "anti-box III" are located on the mRNA track on the 30S ribosomal subunit (Makrides, 1996). Considering that box III might interact with the ribosome during the translation initiation step, it was postulated that this box could act synergistically with the PL sequence in binding PAP mRNA to the ribosome. Box IV is located further down from the typical "downstream box" area in mRNA and it may also be involved in auxiliary interactions during formation of the translation initiation complex. The longer distance from the AUG codon, however, could favour its interaction with the corresponding complementary domain in 16S rRNA without disturbing the accommodation of the initiation codon into the P site.

Thus far, the "downstream box" phenomenon has been studied on either SD-containing (Sprengart *et al.*, 1996) or leaderless (McCarthy & Brimacombe, 1994) mRNAs, but not on mRNAs containing alternative (non-SD) translational initiators. Since the "downstream box" exists in both PL- and SD- containing PAP mRNAs, the higher level of expression obtained with the PL sequence may be explained by a better synergism between the upstream and downstream ribosome binding sites in comparison with the conventional SD sequence. An alternative explanation of the lower efficiency of the SD sequence in this study might be that the *Bam*HI site (placed in front of the initiation codon) includes the sequence GAUCC, [called translation promoting sequence or TPS (Resch *et al.*, 1996)]. This sequence is complementary to nucleotides 1529-1533 in 16S rRNA (see Fig.27) located immediately upstream from the "anti-SD" binding site (1534-1540). Thus, the upstream ribosome binding region for the PAP mRNA carrying a consensus SD sequence is extended an additional 5 nt yielding a total of 13 bases complementary to the 3' end of 16S rRNA. It seems likely that the extremely low free binding energy ($\Delta G = -21.4$ Kcal/mol) in this case is far away from the optimal value required for an efficient translation initiation. This TPS, in conjunction with the downstream box III, may also be responsible for the residual expression of PAP when both boxes (I and II) were mutated (Fig.28, Table 9).

The high efficiency of PL as a translational enhancer may also be explained by the interactions between mRNAs and ribosomal proteins. It was reported that *E. coli* ribosomal protein S1 has been shown to bind to the TMV leader sequence (Tzareva *et al.*, 1994) and also suggested to interact with the *Mycoplasm tuf* enhancer (Ringquist *et al.*, 1995). S1 protein can bind to U-rich mRNA sequences or recognize specific RNA structures, which may allow

ribosome binding in the absence of an SD interaction. In other words, S1 acting as a bridge to facilitate contacts between the mRNA and small ribosomal subunits may account for the observed stimulatory effects (Sprengart and Porter, 1997).

In addition to the PL sequence from a polylinker, a thirty-three nt fragment derived from the sequence upstream of coat protein gene of potato virus X was also found to initiate translation. Although 5' untranslated regions from several plant viruses (Pugin *et al.*, 1994; Turner *et al.*, 1994 a & b), have been shown to increase expression of heterologous genes *in vivo* and *in vitro*, very few translational enhancer sequences immediately upstream of the CP gene of plant viruses have been reported. In this study, the thirty-three nt fragment was found to enable efficient expression of the PAP_f gene in *E. coli* in the absence of the classical Shine-Dalgarno sequence (approximately 1/3 yield compared to that with SD sequence).

It has been shown previously that the CP of TMV can be synthesized efficiently from both a cell-free system derived from *E. coli* as well as from a chloroplast system (Glover and Wilson, 1982; Camerino *et al.*, 1982). Unlike eukaryotic cell-free systems, initiation of the TMV CP in *E. coli* took place from a region internal to the RNA transcript. Two possible ribosome entry sites complementary to the 3' end of 16S rRNA, were identified immediately in front of the TMV CP (Glover and Wilson, 1982). SD-like sequences have also been identified upstream of the CP gene of Carlaviruses (Foster *et al.*, 1992). To investigate how PAP is expressed without the SD sequence, the complementarity of the 5' untranslated mRNA of PAP and the 16S rRNA was analysed by the computer software program SEQAID. Two overlapping boxes, located 11 and 15 nucleotides upstream of the AUG start codon of the PVX CP gene were found to be complementary to two nucleotide regions (G^{126}-U^{132} for box A and C^{371}-G^{376} for box B) of the

16S rRNA. To determine whether the complementary boxes were responsible for translation initiation, both boxes were mutated and the yield of expressed PAP was reduced dramatically (0.1 mg/l). Thus, it seems the two boxes function in a similar capacity as the SD sequence. However, considering that the 5' region of the 16S rRNA is buried inside and not localized on the ribosomal surface (Oakes *et al.*, 1990), the two boxes in the mRNA may not bind the complementary sequences in the 16S rRNA. Thus, it appears that mRNA and rRNA complementarity may not be the only reason for the expression. For instance, the complementary boxes in the mRNA may bind to ribosomal proteins during translation initiation.

Resistance of Transgenic Tobacco Plants to Viral Infection

PAP has been demonstrated to have wide range of antiviral activity (Chen *et al.*, 1991). It is therefore logical to use the PAP gene in the development of disease-resistant crops in agriculture. Plant virus diseases cause significant losses to crops worldwide. To improve the yield of crops and produce virus-free plants, some conventional methods have been employed. These include the use of healthy planting materials, eradication of infected plants, weeds and other virus sources, and the use of chemical pesticides to reduce the number of vectors and thus control plant virus diseases. All of these methods, however, have many disadvantages and are often inefficient (Hull and Davies, 1992). Transformation of plants with viral coat protein genes that interfere with the viral infection cycle has proven to be a successful method for the control of plant viral disease, but protection against other unrelated viruses is usually poor (Stark and Beachy, 1989; Xu, H., 1995).

This study describes the introduction of resistance to TMV infection in tobacco and

suggests that two C-terminal deletion constructs of the *PAP* gene (pGA-PAPΔ36, pGA-PAPΔ62, 36 and 62 aa were removed from the C-terminus, respectively) provide some protection against TMV infection.

Since the N-terminal deletion of the PAP gene was found to be lethal to *E. coli* cells, only C-terminal deletion PAP mutants were used. Four forms of the PAP gene [pGA-PAP (full-length), pGA-PAPΔ36, pGA-PAP62, pGA-PAP-161] were transferred into tobacco plants. It was found that the number of putative transgenic shoots regenerated was proportional to the number of amino acids deleted from the C-terminus. Two controls without the PAP gene (no kanamycin in the medium, binary vector devoid of PAP gene) and deletion of 131 aa (no antiviral activity) produced similar numbers of transformants (242, 250 and 265 respectively, see Table 10). However, both the full-length and 36 aa deleted constructs generated fewer transformants (22 and 30 respectively), while the construct with the 62 aa deletion yielded an intermediate number of transformants (164). Thus, it appears that PAP had some degree of toxicity to tobacco plants (though not proven yet), i.e., mutants with larger deletions were less toxic. It is reported that PAP is very abundant in pokeweed plants (10 mg/100 g fresh weight) but results from Lodge *et al.* (1993) revealed that the expression levels of PAP in transgenic tobacco or potato plants were extremely low (10 ng/1 mg of total plant protein). It is thought that the lack of toxicity observed in pokeweed occurs because the native PAP is translated in a pre-form (inactive form) and transported outside of the cytoplasm, then trapped within the cell walls where it is activated by cleavage (Ready *et al.*, 1986). The N-terminal signal peptide seems to play an important role during the transport process and it is possible that the low expression of PAP in transgenic plants may be due to the reduced efficiency of the N-terminal signal to transport this protein. Results

from Chen *et al.* (1993) support the idea that the N-terminal signal may not function properly in other species.

At least 20 transformants from each construct were transferred to rooting media containing 100 μg/ml kanamycin and most of them produced roots. The transformation protocol resulted in morphologically normal shoots but gave rise to transgenic plants which were visually different from the untransformed plants grown under similar conditions, i.e., transformants from constructs pGA-PAP and pGA-PAP-Δ36 generated smaller plants. All putative transgenic plants were first screened by an antiviral activity assay. Plant lines which produced significantly fewer local lesions than the control (pGA643), were selected for further immunoblot analysis. Results from Western blot revealed that only one construct (pGA-PAPΔ36) produced a detectable level of PAP (Fig.33) while PAP in all other constructs including the full-length PAP gene was not detectable (Table 11).

Antiviral activity assays confirmed that five transgenic lines containing detectable levels of PAP were protected from viral infection, while plant lines transformed with the same construct with little PAP expression did not confer any antiviral resistance, suggesting that PAP is responsible for the antiviral activity (Fig.34, Table 11).

The real mechanism of PAP-mediated resistance in transgenic plants is presently unknown (Lodge *et al.*, 1993). However, a certain number of hypotheses about this mechanism have been proposed. Since the inhibitory effect of PAP does not act directly on the virus (Tomlinson *et al.*, 1974), it has been assumed that the antiviral effect is mediated through the inhibition of host cell protein synthesis (Stevens *et al.*, 1981). PAP is predominantly located in the cell wall matrix of pokeweed plants (Ready *et al.*, 1986) and this finding has been incorporated into a hypothesis which states that local damage to the leaf caused by insect vectors releases PAP into the cytosol,

resulting in local plant cell death at the site of infection. Evidence supporting this hypothesis also comes from viruses that are transmitted by aphids but not by mechanical means. Numerous plant viruses are transmitted by mechanisms that do not involve cell breaching. Insect vectors (aphids) may deliver viruses directly into plant vascular tissue, and the virus may spread symplastically via plasmodesmata. The viruses which commonly infect pokeweed, such as pokeweed mosaic virus (Shepherd *et al.*, 1969) and cucumber mosaic virus (Bhargava, 1951) are transmitted by aphids. Recent results (Lodge *et al.*, 1993) confirmed that transgenic plants with the PAP gene are resistant to viruses transmitted by mechanical means but not to those transmitted by aphids. However, work in which rRNA depurination was assayed has shown that this is not the case for six species of dicotyledonous plants (Kataoka *et al.*,1992; Prestle *et al.*, 1992). In contrast, there is strong evidence that cereal seed ribosomes are resistant to the action of endogenous RIPs based on results from *in vitro* resistance, while cereal RIP genes do not encode precursor forms of the RIP (Leah *et al.*, 1991; Walsh *et al.*, 1991). Such RIPs are presumably, cytosolic making it imperative that the ribosomes exhibit resistance to RIP-catalysed depurination. Alternatively, PAP may prevent virus from entering cells by binding to the virus or a component of the cell wall (Lodge *et al.*, 1993). It is also possible that selective internalization of PAP in virus-infected cells, a mechanism believed to occur in mammalian tissue, exists in plants. PAP has been shown to be selectively internalized in virus-infected human cells *in vitro* by a process involving changes in membrane permeability (Fernandes-Puentes and Carrasco, 1980). In addition, all RIPs are known to be more toxic to virus-infected cells than to uninfected cells. Of particular interest is evidence that some RIPs inhibit HIV gene expression without inhibiting the host cell gene expression, suggesting the antiviral mechanism may go beyond nonspecific translation interference

and may be directly interacting with viral nucleic acids (McGrath *et al.*, 1989).

Different from the above construct (pGA-PAPΔ36), three lines transformed with pGA-PAPΔ62, which had no discernable levels of PAP, also conferred a reduced antiviral activity (Table 11). As mentioned before, the expression level of this construct in *E. coli* expression system was very low (one tenth compared to that of the full-length PAP). It is very likely that the antiviral activity resulted from a level of PAP protein that is below the detectable limit. However, another possibility may be that the antiviral activity was mediated by PAP mRNA. This seems less likely because TMV mixed with *in vitro* transcribed PAP mRNA did not inhibit TMV infection based on a local lesion assay (data not shown).

It was surprising that the full-length PAP_f in the transgenic plants was not detected and the transgenic plants were not resistant to viral infection. Transgenic tobacco expressing the full-length PAP gene has been reported to be resistant to viral infections (Lodge *et al.*, 1993), even though PAP expression is reported to be extremely low. The only difference between the full-length PAP gene in this study and the one reported by Lodge *et al.* (1993) was the 5' untranslated region or ribosome-binding site. Lodge *et al.* (1993) used the natural 5' untranslated region of the PAP gene isolated from the pokeweed plants whereas for this study the sequence was derived from the ribosome-binding site of the coat protein (CP) of PVX. Possibly the sequence from PVX was too strong such that only transgenic lines with mutated (e.g. nonsense mutation) PAP genes were isolated.

Since the above antiviral results were from the first generation of transgenic plants, it would be essential to analyse the segregation of the transgene and the phenotype of the next generations. However, possibly due to the toxicity of PAP, the transgenic plants which conferred

the highest antiviral resistance were sterile and did not produce any seeds. Lodge *et al.*(1993) also obtained sterile transgenics and proposed a similar explanation.

The production of genetically engineered crops for commercial purposes is an attractive and efficient alternative for managing future food production (Horsch, 1993). The work presented here suggests that a C-terminus truncated PAP gene (pGA-PAPΔ36) can be successfully transformed into tobacco plants and provide resistance against virus infection. Transgenic plants with the PAP gene have advantages compared to other viral genes. First, there is no concern about "superviruses" being produced between natural viruses and viral transcripts if viral genes are transformed in transgenic plants. Secondly, previous methods for creating virus-resistant plants have been specific for a particular virus or closely-related viruses. In order to protect plants against multiple plant pathogens, multiple genes must be introduced and expressed in a single transgenic line. PAP offers the possibility of developing resistance to a broad spectrum of plant viruses by expression of a single gene (Lodge *et al.*, 1993). Thirdly, some RIPs have shown activity toward ribosomes from distantly related species including fungi (Stirpe and Hughes, 1989). For example, purified barley RIP inhibits the growth of fungi *in vitro* (Leah *et al.*, 1991). Expression of the barley RIP cDNA under the control of a wound-inducible promoter in transgenic tobacco plants has also been demonstrated to confer protection against the soil-borne pathogen *Rhizoctonia solani*, as judged by height differences between control and transgenic plants grown in infested soil, although direct measurements of the effect of the transgene on lesion size and fungal growth were not reported (Logemann *et al.*, 1992).

The expressed PAP in transgenic crops may be toxic to humans. However, at least three lines of evidence show that PAP in transgenic plants may be harmless: 1) PAP is not highly toxic

when it is outside the cell because, unlike type II RIPs which have another polypeptide chain for binding to the cell membrane for internalization, PAP does not have a cell-binding chain and shows very little toxicity due to its inability to enter cells. 2) the expression level of PAP in transgenic plants is low. Lodge *et al.* (1993) reported that the PAP concentration obtained from transgenic plants is less than 20 ng per mg of total soluble protein. The results of this study reveal that transgenic plants were obtained only with the truncated forms of the PAP gene and the truncated PAP concentration was extremely low too. 3) Many food plants (such as wheat, barley, corn etc.) contain similar RIPs. Therefore, transgenic crops with the *PAP* gene may provide a very promising means of conferring resistance to plant pathogens.

Testing for Resistance of Transformed CD_4^+ Cells with $PAP_{\Delta N}$ Gene to HIV-1 Infection

PAP, PAP immunotoxin (PAP conjugated with monoclonal antibodies), nucleotide analogs and HIV-1 protease inhibitors have been widely used in clinical trials or in treating AIDS patients, and promising results have been obtained (Barbieri *et al.*, 1993; Kent and Rud, 1996; Lea and Faulds, 1996 and Jadhav *et al.*, 1997). However, all these agents are facing a challenging problem: HIV-1 mutates so fast that these drugs become less and less efficacious. To fundamentally solve the mutation problem, other approaches such as gene therapy are being developed (Harrison *et al.*, 1991 and Dropulic and Jean, 1994). The principle of the strategy for this study is to use two early regulatory proteins, Tat and Rev, for the selective expression of the PAP gene and therefore to achieve selective killing of HIV-1 infected cells.

To ensure the expressed PAP can kill virus-infected cells, the effect of *E. coli*-expressed PAP alone or PAP co-infected with retroviruses on human NIH 3T3 cells was tested. All results

demonstrated that PAP co-infection with retroviruses had a much higher toxicity to cells, i.e., concentrations of 5 μg/ml PAP resulted in 100% death of cells infected with retroviruses but only 25% without retroviruses. Lower concentrations of PAP (less than 0.05 μg/ml) showed lower toxicity (Tables 12 &13). A similar observation was reported in cells infected with other viruses (Ussery *et al.*, 1977; Barbieri and Stirpe, 1982; Fao-tomasi *et al.*, 1982). The mechanism by which PAP kills virus-infected cells at much lower concentrations is not clear. It is proposed that binding of PAP to the virus *in vitro* may facilitate its entry into cells. There is evidence of weak ionic binding of PAP to various animal viruses such as the influenza virus (Tomlinson *et al.*, 1974) as well as to plant viruses including cucumber mosaic virus (CMV) (Tomlinson *et al.*, 1974) and tobacco mosaic virus (TMV) (Kumon *et al.*, 1990). It is also possible that viruses may increase the cytoplasmic concentration of PAP by increasing membrane permeability to allow PAP to enter the cell. The plasma membrane is generally a barrier against invading viruses and is relatively impermeable to macromolecules. Adsorption of various DNA and RNA animal viruses to susceptible cells can be accompanied by an increased fluidity of lipids in the host cell membranes (Fernadez-Puentes and Carrasco, 1980; Kohn, 1979; Levanon and Kohn, 1977).

The retroviral vector pUCMoTN-Ti-Ri-Ter is a pUC-based, Moloney murine leukemia virus-derived retroviral vector. Tat and Rev are two HIV derived regulatory proteins that have been demonstrated to be absolutely essential for viral replication (Cullen, 1991). It has been reported that the basal expression of toxic genes such as diphtheria toxin A chain (DT-A) gene was greatly reduced from the HIV-1 LTR in the absence of Tat and reach maximal expression in the presence of Tat (Harrison *et al.*, 1991). Consequently, selective suicide of HIV-infected cells should occur before viral progeny are produced (Harrison *et al.*, 1992). The Tat- and Rev-

inducible retroviral vectors used in this project were developed and successfully used to inducibly express *trans*-dominant mutants of HIV-1 proteins to confer anti-HIV-1 resistance (Liem *et al.*, 1993).

To ensure that expression of a toxic gene was limited to HIV-1 infected cells, the N-terminal signal peptide (22 aa) of PAP was deleted. This signal peptide is considered to transport inactive PAP precursor from the cytoplasm to the outside of cell membrane where it is cleaved and activated (Ready *et al.*, 1986). The active form of the PAP gene (without signal peptide) used for anti-HIV-1 gene therapy under Tat- and Rev-inducible expression was expected to restrict PAP production to the HIV-1 infected cells whose destruction was desired.

To determine the cell killing ability (ribosome-inactivating activity) of the PAP cassette in the retroviral vector, the PAP cassette containing the Kozak consensus ribosome-binding site (Kozak 1989) and PAP gene was subcloned into pBS^{+} plasmid for *in vitro* transcription and translation analyses. Results (Fig.39) showed that the PAP gene partially inhibited *in vitro* protein translation, indicating that the PAP gene was biologically active. To investigate the cause of the partial but not complete inhibition, the entire open reading frame of the PAP gene in the retrorviral vector was sequenced. The results demonstrated that the A^{561} (core region of PAP) was deleted resulting in premature termination of translation of the PAP gene. Mutation of the PAP gene in the retroviral vector may suggest that the *E. coli* cells could not tolerate the leaky expression of the N-terminal signal deleted PAP gene but could tolerate the truncated one (A^{561}) under the Ti promoter. It is very likely that the partial but not complete inhibition of protein synthesis in the *in vitro* translation assay may be due to the low ribosome-inactivating activity resulting from premature termination caused by the A^{561} deletion.

The two retroviral vectors either with or without the PAP gene were, therefore, transfected into the packaging cell line Psi-2 and finally the PAP gene was integrated into target cells (human CD_4^+ lymphocyte-derived MT4). PCR analysis revealed that the PAP gene was integrated into the cellular genome (Fig.36) and the RT-PCR data also confirmed the presence of PAP mRNA in the cytoplasm (Fig.37). However, HIV-1 challenge experiments indicated no difference in HIV-1 yield (p24 as indicator of HIV-1) between the MT4 cells containing the PAP gene and those devoid of it. This result indicated that PAP does not confer resistance against HIV-1 infection. To confirm whether the PAP gene in the cellular genome was mutated and non-functional due to the toxicity, cells (GP+E cell line) which constitutively expressed Tat and Rev proteins were infected with vector particles containing the PAP gene. If the PAP gene was functional, the cells would be killed due to the inducers, Tat and Rev, otherwise, the PAP gene was not functional. Result from this experiment showed no inducible killing of cells was observed (data not shown), indicating that the PAP gene was not functional. These results suggest that the N-terminal deleted PAP gene may be too toxic and may not be suitable for anti-HIV-1 gene therapy.

The question arises: why did the prematurely terminated PAP gene from the retroviral particles inactivate the ribosomes *in vitro,* but was unable to confer resistance to HIV-1 in cells? It has been reported (Caruso *et al.*, 1992) that a stable integration of the Tat- and Rev-controlled diphtheria toxin A chain (DT-A) gene is not compatible with cell viability. Caruso *et al.* (1992) found fewer clones with the DT-A gene compared to the control lacking the DT-A gene after transfection, and further analysis of 20 clones by Southern blot hybridization showed that all were negative for the DT-A gene. Results from Caruso *et al.* (1992) suggested that 1) *Tat-* and *Rev-*control was not absolutely inducible, 2) a low "leaky" level of DT-A expression was toxic to

mammalian cells and 3) recombination of DT-A gene took place due to the toxicity. It is very likely that the wild-type PAP protein in this study was too toxic and only non-functional PAP mutants were isolated which failed to provide resistance to HIV-1. Thus, less toxic forms of the PAP gene (e.g., C-terminal truncated forms of the PAP gene) may be tested in future studies. In addition, it was recently reported that a nonsense suppression may be used to control the expression of toxic genes in mammalian cells (Robinson and Maxwell, 1995). In this novel strategy, a leaky termination codon (TAG) was introduced into the DT-A gene so that the DT-A activity was eliminated. However, introduction of a serine inserting suppressor tRNA restored the DT-A expression. It is possible that the same strategy may apply to the PAP gene.

FURTHER EXPERIMENTS

Future work related to that described in this thesis may be carried out. Work in transgenic plants may be continued. The results of this study suggested that transgenic tobacco plants transformed with C-terminal deletion constructs of the PAP gene (pGA-PAPΔ36 and pGA-PAPΔ62) conferred resistance to TMV infection. It would be interesting to see if these plants are resistant to other pathogens (other viruses, bacteria or fungi) as well. The above C-terminal deleted constructs can also be transformed into other economically important crops such as potatoes and tomatoes etc.

It is of interest to further study the structure-function relationship of the PAP protein by using mutational analysis in order to identify the critical amino acids required for PAP enzyme activity.

The PAP gene devoid of N-terminal signal codons did not render cells resistant to HIV infection. To confirm the PAP gene in the cellular genome is mutated and non-functional, the PAP gene in the genome may be amplified and sequenced. If it is mutated due to its toxicity, less toxic forms of the PAP gene such as C-terminal deleted forms of the PAP gene or the full-length PAP gene including the N-terminal signal peptide should be tested for the construction of retroviral vectors for anti-HIV infection. In addition, more strictly controlled inducible promoters should be developed.

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APPENDICES

A. Oligonucleotides used in this thesis

P_{PAPF} 5'GAT-GGATCC-GATGAAGTCGATGCTTGTGGTGAC3'

P_{PAPR} 5'CGC-GGATCC-T-GGTACC-TTGCATCATAATTGGAAGTTTTATTTC3'

P_{SDF} 5'TCGACAAGGAGGTTTG3'

P_{SDR} 5'GATCCAAACCTCCTTG3'

P_{PVXF} 5'CTAGATTGAACGGTTAAGTTTCCATTGATACTCGAAAGATGAAGT3'

P_{PVXR} 5'CGACTTCATCTTTCGAGTATCAATGGAAACTTAACCGTTCAAT3'

$P_{\Delta N}$ 5' GAT-GGATCC-GATGGTGAATACAATCATCTACAAT 3'

$P_{\Delta CF}$ 5' TCGAGCTATGATGAAGATCTT3'

$P_{\Delta CR}$ 5' CTAGAAGATCTTCATCATAGC3'

P_{PstI} 5'GTAAA-CTGCAG-GCATCGTGGTGTCA5'

PM_{boxI} 5'TCTAGA-AGATCT-TTAATA-AGTAGGTTCAAATTTA3'

PM_{boxII} 5' TAGA-AGATCT-AACTCGA-TATTATA-CAAATTTATGAA3'

$PM_{boxI\&II}$ 5' TCTAGA-AGATCT-TTAATAATATTATA-CAAATTTATGAATCTATTATACAG3'

PM_{PVX} 5'CTAGTCTAGATTAAACGGTTA-TATATAATATA-ATACTCGAAAGATGAAG3'

$P_{HIV\Delta NF}$ 5' CCCCCGGGCCAAGTCGGCC-GCCACCATGGTGAATACAATCATCTACAAT3'

$P_{HIV\Delta NR}$ 5'TTCCCCCG-GGCCAAGTCGGCC-TTATGATCAGAATCCTTCAAATAGA3'

$P_{HIV\Delta PAPF}$ 5' ATCGATCCGGATCCTCGCGAGCGGCCGCACT3'

$P_{HIV\Delta PAPR}$ 5'GCGGCCGCTCGCGAGGATCCGGATCGATACT3'

P_{Hybrid} 5' GATATTGTCACCACAAGC 3'

Ppapseq1 (P_{EcoRI}) 5'ATAGGCGTATCACGAGG3'

Ppapseq2 (Pm1) 5'TGGAAGTACCACCATTAGCA3'

Ppapseq3 (X9) 5'ACACTAATGCTGAGAC3'

Ppapseq4 (X10) 5'ATCAAGAAGTCAAGTCC3'

Ppapseq5 (Pm-2) 5'TCAAGTACATAGAGAATCAG3'

Ppapseq6 (Pm-3) 5'TGCCAAGTGGATAGTGTTGA3'

P_{SphI} 5' AGGTTGAGGCCGTTGAGCA 3'

P_{RBSF} 5' CTAGATTGAACGGTTAAGTTTCCATTGATACTCGAAAGATGAAGT 3'

P_{RBSR} 5' CGACTTATCTTTCGAGTATCAATGGAAACTTAACCGTTCAAT 3'

B. Growth Media for Bacteria

a) YT Broth (2 litres)

16 g Bacto-tryptone
10 g Yeast extract
10 g NaCl
pH 7.0
Make 2x YT and add 1.5 % agar for plates.

b) Terrific Broth (TB) (1 litre)

Solution A (900 ml):
12 g Bacto-tryptone
24 g Yeast extract
4 ml of glycerol

Solution B (100 ml):
2.3 g KH_2PO_4 (Potassium phosphate monobasic)
12.5 g K_2HPO_4 (Potassium phosphate dibasic)

Autoclave separately, cool to room temperature and mix solution A and B.

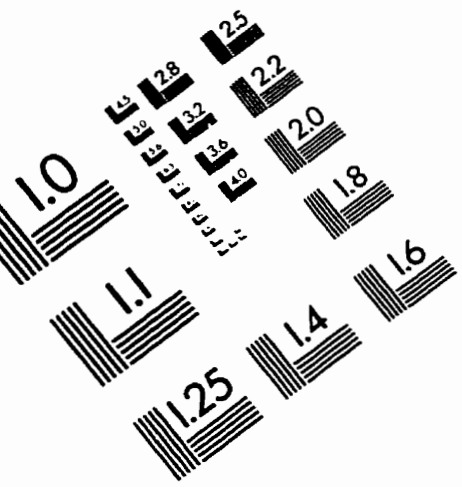
C. Media for Tobacco Tissue Culture

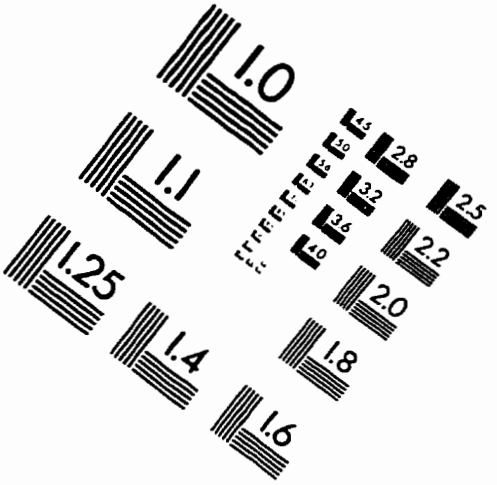
MS41:
4.4 g MS medium (catlogue No. 6899, Sigma)
3% sucrose
1 mg/L BAP
0.4 mg/L NAA
9 g/L agar
pH 5.8

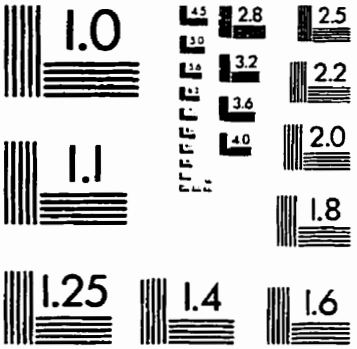
MS41a:
MS41
100mg/L kanamycin
400 mg/L carbenicillin

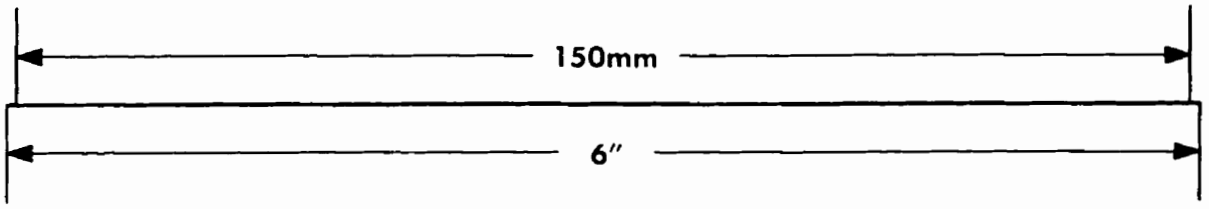
MS42:
2.2 g MS medium (catologue No. 6899)
1.5% sucrose
100 mg/L kanamycin
400 mg/L carbenicillin
pH 5.8

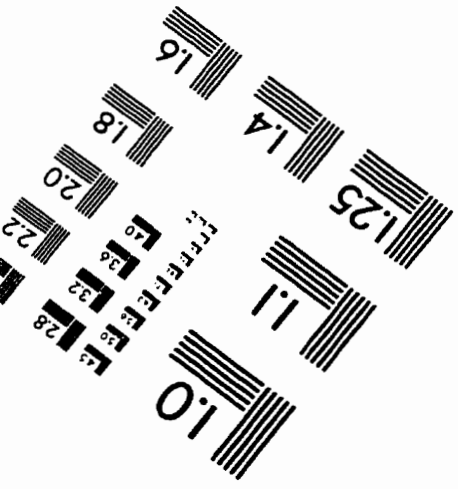
IMAGE EVALUATION
TEST TARGET (QA-3)

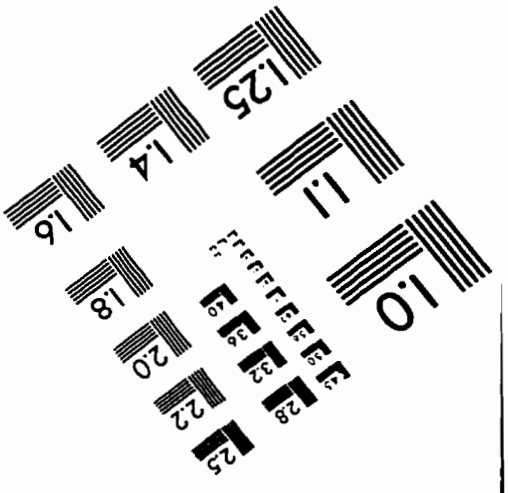












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