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Purification, characterisation and cDNA sequence of tRNA nucleotidyltransferase from *Lupinus albus.*

Kandavel Shanmugam

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ABSTRACT

Purification, characterisation and cDNA sequence of tRNA nucleotidyltransferase from *Lupinus albus*

Kandavel Shanmugam

The enzyme ATP(CTP):tRNA-specific tRNA nucleotidyltransferase is required for the synthesis of functional tRNAs in eukaryotic cells. In yeast a single gene provides this enzyme activity for both nuclear/cytoplasmic and mitochondrial tRNA biosynthesis. To determine if this is also the case in plants we isolated tRNA nucleotidyltransferase from lupin. Characterization of the purified protein revealed an apparent molecular weight of 64 kDa, similar to the yeast enzyme. It also showed pH and temperature optima similar to the yeast, and *Lupinus luteus* enzymes. The purified protein was subjected to tryptic digestion and the amino acid sequence of two peptides determined. Based on these amino acid sequences two oligonucleotides were designed and used to direct polymerase chain reaction (PCR) on the lupin cDNA library. Using the single PCR product generated as a hybridisation probe 16 independent cDNA clones were isolated. Nested deletions were done on both strands of the longest of the 16 clones and the complete cDNA sequence determined. The predicted amino acid sequence consisted of a total of 560 residues and showed similarity to the yeast protein. This protein has been identified as *L. albus* tRNA nucleotidyltransferase based on its enzymatic characteristics and its similarity to the yeast protein. With this cDNA in hand we are now in a position to study the intracellular localisation of this protein in plants.

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ABBREVIATIONS

bp	Base pairs
cDNA	Copy deoxyribonucleic acid
cpm	Counts per minute
EDTA	Ethylenediaminetetraacetic acid
g	Gram
kbp	Kilo base pairs
kDa	Kilo Daltons
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
tRNA	Transfer ribonucleic acid
rpm	Revolutions per minute
BSA	Bovine serum albumin
CnBr	Cyanogen Bromide
nm	Nanometers
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethyleneglycol
pfu	Plaque forming units
TEA	Tris, EDTA acetate buffer
SDS	Sodium dodecyl sulphate
Xg	Times gravity

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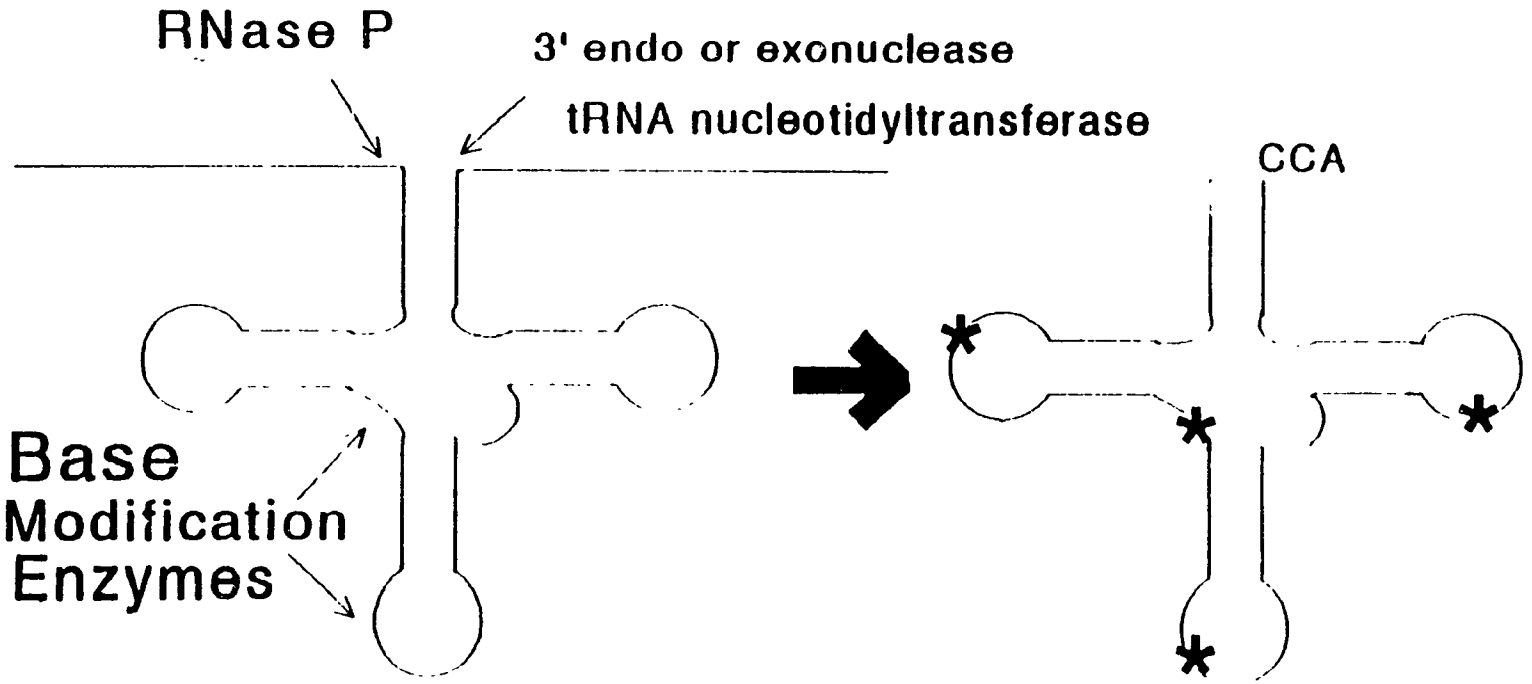
I. INTRODUCTION

In prokaryotes transcription and translation are coupled. Thus, the translational machinery is not separated from the transcriptional machinery so that translation begins even before transcription is completed. In a eukaryotic cell, transcription and translation occur in separate cellular locations. Transcription takes place in the nucleus while translation takes place in the cytoplasm. Hence, transfer RNAs which are transcribed from the nuclear DNA have to be exported to the cytoplasm where they function in protein synthesis. Before tRNAs can function in protein synthesis they have to be processed from a precursor into a mature form. These precursor tRNAs, the products of transcription of tRNA genes, contain the complete tRNA primary sequence as well as additional residues at both the 5' and 3' ends. The maturation of a functional tRNA (Fig. 1) is a multistep event which involves 5' and 3' cleavages catalysed by processing enzymes, as well as intron removal (if necessary), 3'-terminal CCA addition by tRNA nucleotidyltransferase and specific base modifications. Because many of the steps of tRNA maturation take place in the nucleus and only mature or nearly mature tRNAs can exit the nucleus many of these enzymes have to be imported from the cytoplasm, where they are synthesised, into the nucleus where they function. This is also the case with RNA polymerase that is responsible for the production of the primary tRNA transcripts.

We are interested in how these enzymes, synthesised on cytoplasmic ribosomes, enter the nucleus to perform their respective functions. The nucleoplasm

FIGURE 1

tRNA Biosynthesis



is, however, not the only place in a eukaryotic cell where tRNAs can be found. Both mitochondria and chloroplasts contain their own tRNA genes which are expressed to produce tRNAs which function in organellar protein synthesis. The maturation of tRNAs follows a similar path in these organelles as it does in the nucleocytoplasm and, similarly these tRNA maturation enzymes must be imported from the cytoplasm into these organelles. Although maturation of tRNAs shares many common features in terms of the enzymes that are involved these enzymes themselves may differ in their physical and biochemical properties among different organisms and organelles. Therefore, the maturation of tRNA from prokaryotes and eukaryotes are discussed separately below.

1. MATURATION OF tRNA's

A) PROKARYOTES

Almost 42 % of *E. coli* tRNA genes are present as clusters on the chromosome (King *et al.*, 1986). All tRNA genes are transcribed to produce precursor tRNA's, which in *E. coli* are about 130 nucleotides in length (Deutscher, 1988). These precursors need to be processed at the 5' and 3' temini of the tRNA itself to give a mature tRNA (King et al., 1986). In *E. coli*, the enzyme that cleaves the leader sequence to produce a mature 5' terminus is an endonuclease, RNase P (Kole and Altman, 1979). Although this enzyme can recognise tRNA substrates at different stages of processing, tRNAs with a processed 3' terminus have been shown to be the best substrates (Altman, 1984). Maturation of the 3' terminus of tRNAs

in *E. coli* takes place exonucleolytically and there seems to be a specificity in the processing since processing takes place until the exonuclease encounters a double stranded region in the tRNA at the 3' terminus of the partially processed tRNA (King *et al.*, 1986). Cudny and Deutscher (1980) showed that maturation at the 3' end of tRNA was carried out more efficiently by RNase D than RNase II in that RNase D generated a correct 3' terminus and RNase II removed two additional nucleotides. Deutscher and Evans (1977) showed that the extra nucleotides removed by RNase II were replaced by tRNA nucleotidyltransferase, although a mutant deficient in tRNA nucleotidyltransferase did not show any detectable alteration in tRNA processing (Deutscher and Evans, 1977). Although RNase D seemed to be the major 3' processing enzyme in *E. coli*, a deletion mutant of the RNase D gene was still viable suggesting that there could be other activities that can replace RNase D for 3' processing (Asha *et al.*, 1983). In *E. coli* the 3' CCA terminus is encoded by the respective tRNA genes (Komine *et al.*, 1990). In *E. coli* the enzyme, tRNA nucleotidyltransferase, is encoded by the CCA gene which is located at 66 minutes in the *E. coli* chromosome (Cudny *et al.*, 1986). Although the primary tRNA transcripts in *E. coli* contain the entire mature tRNA molecule, other prokaryotes including *B subtilis* and even some *E. coli* phages contain incomplete 3' ends to which CCA has to be added post- transcriptionally (Asha et al., 1983). Therefore, tRNA nucleotidyltransferase plays an essential role in the production of mature tRNAs in these organisms.

B) EUKARYOTES

As in prokaryotes, the synthesis of eukaryotic tRNAs involves production of a precursor tRNA transcript, processing at the 5' and the 3' termini of precursor tRNA, base modifications and addition of CCA to the processed 3' terminus. Unlike most prokaryotes, some eukaryotic tRNA genes contain introns which must also be removed to form a functional tRNA (Abelson, 1979).

i) PROCESSING

Processing events involved in the maturation of precursor tRNAs in eukaryotes involve removal of the 5' leader sequence and removal of the 3' trailer sequence. Removal of the 5' leader sequence from precursor tRNAs is known to be catalysed by an endonuclease (Castano *et al.*, 1986). The entire leader sequence is removed as one piece which results in the production of a mature 5' terminus (Castano *et al.*, 1986). Though the endonuclease RNase P is an RNA-containing enzyme in eukaryotes like *X. laevis* and yeast, ribozyme activity (*i.e.*, catalysis by the RNA component of the enzyme) has not been demonstrated (Castano *et al.*, 1986). An equivalent activity designated 5' pretransferase was seen in the oocytes of *X. laevis* in which no RNA cofactor was detected (Castano *et al.*, 1986). In eukaryotes processing of the 3' terminus, *i.e.*, removal of the trailer sequence, is catalysed by an endonuclease (Garber and Gage, 1979). This contrasts the situation in *E. coli* where an exonuclease removes the entire trailer. In *X. laevis*, the endonuclease that processes the 3' end of tRNA seems to be a single polypeptide and requires a 5' processed tRNA transcript as its substrate (Castano *et al.*, 1985). In most eukaryotes

5' processing preceeds 3' processing though this is not true *in vitro* (Manam and VanTuyle, 1987).

ii) SPLICING

Introns are removed by a process called RNA splicing (Abelson, 1979). Unlike the introns in eukaryotic mRNAs which possess partial conserved sequences at the splice sites, there is no conservation of primary sequence in the case of tRNA introns (Abelson, 1979). Hence, there is a likelihood of having many ways of tRNA splicing among the different classes of precursor tRNAs. Out of 400 tRNA genes in yeast at least 40 of them have introns varying in length from 14 to 60 nucleotides (Guthrie and Abelson, 1982). The introns in tRNAs are identical among the same family of genes but vary between families.

Compared to yeast and other vertebrates not much is known about tRNA splicing in plants (Stange and Beier, 1987). Stange and Beier (1987) showed that in the case of maturation of pre-tobacco $tRNA^{Tyr}$ using S_{23} and S_{100} wheat germ extracts, processing of the flanks precedes intron excision while base modifications occur in both intron containing pre-tRNA with mature ends and in mature tRNA. In contrast to the above statement, in *in vitro* splicing systems like HeLa cell nuclear extracts, intron excision preceeds processing of the flanking ends (Stange and Beier, 1987). In yeast it has been shown that as with intron removal from mRNA precursors, introns are spliced out of tRNAs prior to the tRNA leaving the nucleus (Clark and Abelson, 1987).

iii) BASE MODIFICATIONS

Base modifications take place during tRNA maturation resulting in modified nucleosides derived from adenosine, guanosine, cytidine and uridine (Nishimura, 1979). Although these modifications are not essential for cell viability (Bjork *et al.*, 1987) they seem to play an important role in normal tRNA function. Modification of specific nucleosides in the tRNA molecule may increase translational efficiency and fidelity and also help in stabilising the conformation of a tRNA (Bjork *et al.*, 1987). It has also been shown that more than one enzyme can catalyse the formation of the same modified base in the different tRNAs (Bjork *et al.*, 1987).

iv) CCA ADDITION

Finally, prior to becoming a functional tRNA molecule, the post-transcriptional addition of 3' terminal cytidine, cytidine and adenosine (CCA) to the 3' terminus of a tRNA is required. Unlike *E. coli*, where the CCA is encoded by the respective tRNA genes, eukaryotic tRNA's have the CCA added at the processed 3' terminus post-transcriptionally by the enzyme ATP:CTP tRNA-specific tRNA nucleotidyltransferase (Aebi *et al.*, 1990).

C) MATURATION OF CHLOROPLAST tRNAs

Although chloroplasts are thought to be of prokaryotic origin, they differ from prokaryotes in some ways (Gray and Doolittle, 1982). In contrast to bacterial tRNA's, which are normally clustered and transcribed in a polycistronic fashion,

plant chloroplast tRNA genes are not clustered (Shinozaki *et al.*, 1986). Yamaguchi *et al.* (1987) showed that although maturation involves removal of the 5' leader sequence by RNase P similar to what occurs in *E. coli*, the cleavage of the 3' trailer is catalysed by a specific endonuclease in contrast to the exonuclease responsible for 3' trimming in *E. coli*. Complete sequencing of the tobacco chloroplast genome (Shinozaki *et al.*, 1986) showed that in one third of the tobacco tRNA genes, the potential first C of the 3' CCA is encoded by the respective gene such that maturation could be completed with the addition of one cytidine and one adenosine residue. However CA must still be added post-transcriptionally again like eukaryotes and not like *E. coli*. Therefore, tRNA nucleotidyltransferase or some similar activity must be required for chloroplast tRNA maturation. Wang *et al.* (1988) showed that the 3' endonuclease activity is highly specific for a 5'-processed substrate. They also showed that the RNase P of chloroplasts does not have an RNA subunit comparable to that of *E. coli*. Plant chloroplast tRNAs have very long introns ranging from 325-2526 bases (Marechal-Drouard *et al.*, 1993). To date all introns in plant chloroplast tRNAs have been located in the anticodon loop except in the case of a $tRNA^{Gly}$ where the intron is in the D stem (Marechal-Drouard *et al.*, 1993). Splicing of introns in chloroplast is carried out by specific chloroplast factors after 5'- and 3'-processing (Marechal-Drouard *et al.*, 1993).

There is no evidence of genes encoding the enzymes responsible for 5'-processing, 3'-processing, CCA addition or splicing in the completely sequenced chloroplast genomes of tobacco, rice and *Marchantia polymorpha* (Marechal-Drouard

et al., 1993). This suggests that these enzymes are coded for by genes outside the chloroplast genome so that these gene products are subsequently imported into the chloroplast to function.

D) MATURATION OF MITOCHONDRIAL tRNAs

In yeast it has been shown by Palleschi *et al.* (1984) that mitochondrial tRNA genes are mostly transcribed as a cluster. Therefore, there could be a common transcription initiation site upstream of the tRNA gene cluster. This is in contrast to the internal promoters which are characteristic of nuclear tRNA genes (Palleschi *et al.*, 1984). Yeast mitochondrial tRNA genes are transcribed by mitochondrial RNA polymerase which is nuclearly encoded (Levens *et al.*, 1981). The 3' endonucleolytic activity in yeast mitochondria is also nuclear encoded as is the protein subunit of the yeast mitochondrial RNase P (Morales *et al.*, 1992) which functions with a 490 base mitochondrially-encoded RNA (Miller and Martin, 1983). The tRNA genes in human mitochondrial DNA are located in three transcriptional units being transcribed at three different rates (King and Attardi, 1993). These tRNAs are scattered throughout these transcripts and correct 5'- and 3'-processing of these tRNA genes is thought to be responsible for the production of functional mRNA and rRNA molecules (Clayton, 1984). Recently many cellular disorders have been linked to mutations in mitochondrial tRNAs (Bindoff *et al.*, 1993) indicating the role that correct tRNA maturation may play in cell viability. Numerous animal mitochondrial genomes have been sequenced and analysis of these sequences suggest

that, as in yeast, none of the proteins required for mitochondrial tRNA maturation are synthesised from mitochondrial genes. These enzymes must be encoded by nuclear genes, translated on cytoplasmic ribosomes and subsequently imported into the mitochondria where they function.

In plants, mitochondrial tRNA genes are not clustered or closely linked to other genes (Bonen and Gray, 1980). Maturation of plant mitochondrial tRNAs like their nuclear counterparts, requires 5' and 3' processing and addition of CCA at the processed 3' end by tRNA nucleotidyltransferase (Hanic-Joyce and Gray, 1990). As in animal mitochondria there is no tRNA splicing since to date no plant mitochondrial tRNA genes have been shown to contain introns. Hanic-Joyce and Gray (1990) showed that the processing of tRNAs in plant mitochondria can be compared to that which takes place in the chloroplast. Although there appears to be a specific order of events (5' processing followed by 3' processing followed by CCA addition) for maturation of precursor tRNAs *in vitro* in extracts from organisms like *X. laevis* (Castano *et al.*, 1986), Hanic-Joyce and Gray (1990) showed that *in vitro* processing of precursor tRNA's using wheat mitochondrial extracts resulted in the simultaneous presence of leader + tRNA, or trailer + tRNA implying that either the 5' or the 3' processing can be first. Although they have not separated the 5' and 3' processing activities it is possible that they are separate activities as observed in yeast (Hollingsworth and Martin, 1986) and rat liver (Manam and Van Tuyle, 1987) mitochondria.

2. IMPORT

Enzymes that process precursor tRNA transcripts in the nucleus are translated on cytoplasmic ribosomes and so must be imported into the nucleus to carry out their respective functions. Research in the past has shown that though chloroplasts and mitochondria are of eubacterial origin, many genes that encode important functional proteins are absent in their respective genomes (Marechal-Drouard *et al.*, 1993) suggesting that these proteins have to be nuclear-encoded, translated on cytoplasmic ribosomes and imported into these different intracellular compartments. Mitochondrial import of tRNA maturation enzymes has been well documented in yeast. *TRM1* and *MOD5* are two of the well characterised genes that code for tRNA base modification enzymes in yeast. The *TRM1* gene of *Saccharomyces cerevisiae* encodes N^2, N^2-dimethylguanosine-specific tRNA methyltransferase (Ellis *et al.*, 1987). This enzyme catalyses the modification of a specific guanosine residue to N^2, N^2 - dimethylguanosine in nuclear, cytoplasmic and mitochondrial tRNAs (Ellis *et al.*, 1987). *MOD5* is the structural gene that encodes Δ^2 isopentenyl pyrophosphate:tRNA isopentenyl transferase, another tRNA base modifying enzyme in yeast (Dihanich *et al.*, 1987). This enzyme specifically modifies a nucleotide residue adjacent to the anticodon in some nuclear, cytoplasmic and mitochondrial tRNAs. Mutations in the *MOD5* gene which result in non-functional protein abolish its activity in all cellular compartments in yeast suggesting that the products of this nuclear-encoded gene function in multiple destinations in the cell (Martin and Hopper, 1982, Najarian *et al.*, 1987). More recently, experiments performed by Chen

et al. (1992) showed that CCA addition to both cytoplasmic and mitochondrial tRNAs was affected when a yeast strain carrying a temperature-sensitive mutation in the *CCA1* gene (coding for tRNA nucleotidyltransferase) was placed at the non-permissive temperature (37^0C). This result suggests that as with *TRM1* and *MOD5,* there is a single CCA gene that codes for both mitochondrial and cytoplasmic tRNA nucleotidyltransferase in yeast.

Subsequent experiments with the *MOD5* (Gillman *et al.*, 1991), *TRM1* (Ellis *et al.*, 1989) and *CCA1* (Chen *et al.*, 1992) genes have shown that it is possible to abolish the mitochondrial function of these enzymes while maintaining their nucleocytoplasmic function. Most recently mutagenesis experiments on the 5' region of the yeast tRNA nucleotidyltransferase gene to remove the first 9 or 17 amino acids from this protein showed that these amino acids are important for growth that requires mitochondrial respiration, but had no effect on growth that only requires the cytoplasmic enzyme. Based on these results, it was concluded that the first 9 or 17 amino acids at the amino terminus could function as a mitochondrial import signal and when removed this protein could not be imported into mitochondria (Chen *et al.*, 1992). Both mitochondrial and nuclear targeting signals have also been defined on the *TRM*1 product (Rose *et al.*, 1992). These studies have shown not only that these yeast proteins carry information that target them to the nucleus and the mitochondria where they function in tRNA maturation but also that these tRNA modification enzymes carry more than one targeting signal.

3. PROPERTIES OF THE tRNA NUCLEOTIDYLTRANSFERASE GENE

Before this study, tRNA nucleotidyltransferase genes had been characterised only in one prokaryote, *E. coli* (Cudny *et al.*, 1986), and in one eukaryote, yeast (Aebi *et al.*, 1990).

The open reading frame that codes for the *E.coli* CCA gene is 1236 bp in length and codes for a protein with a predicted molecular weight of 46 408 Daltons (Cudny *et al.*, 1986). In yeast, the gene that codes for ATP:CTP tRNA specific-tRNA nucleotidyltransferase was isolated by complementation with a yeast genomic library of a temperature-sensitive strain carrying a mutation in the tRNA nucleotidyltransferase gene (Aebi *et al.*, 1990). The gene shown to complement the ts mutation contains an open reading frame of 1641 base pairs which could code for a protein of 546 amino acids. This protein would have a predicted molecular weight of 62 000 Daltons which is slightly larger than the *E. coli* enzyme. This is in agreement with the size of the purified yeast tRNA nucleotidyltransferase, which is 59 kDa (Chen *et al.*, 1990) based on SDS-PAGE. The amino acid sequence of the yeast tRNA nucleotidyltransferase, predicted from the nucleotide sequence, showed significant similarity to the amino terminal region of the *E. coli* enzyme (Aebi *et al.*, 1990). Aebi *et al.* (1990) also showed that the accumulation of tRNAs without the CCA terminus was greater in the mutant strain than in the wild type and that the accumulation of tRNAs devoid of the 3'CCA terminus became even greater after the mutant strain was shifted to the non-permissive temperature (37^0C). Isolation of a temperature-sensitive strain suggests that there is only one tRNA nucleotidyl-

transferase in yeast (Aebi *et al.*, 1990).

Southern hybridisation of *E. coli* genomic DNA cut with various restriction enzymes and probed with the *E. coli* CCA gene revealed a single positive signal (Cudny *et al.*, 1986). Hence, it was concluded by Cudny *et al.* (1986) that only one copy of the CCA gene is present in the *E. coli* chromosome. Heterologous hybridisations performed on the genomic DNA of *Homo sapiens, Bacillus. subtilis, Petunia* sp., yeast and *Salmonella* using the *E. coli* CCA gene as probe revealed a positive hybridisation only with the *Salmonella* genomic DNA (Cudny *et al.*, 1986). Based on the above, Cudny *et al.* (1986) concluded that tRNA nucleotidyltransferase from different organisms tended to differ from one another at the DNA level.

Mutation at amino acid 70 (Gly to Asp) deprived the *E. coli* enzyme of most of its AMP incorporating activity while the CMP incorporation by the same protein remained normal (McGann and Deutscher, 1980). This mutation was later found to be linked to the AMP incorporating site of tRNA nucleotidyltransferase (Zhu *et al.*, 1986).

4. PROPERTIES OF tRNA NUCLEOTIDYLTRANSFERASE

Among tRNA modifying enzymes in *E. coli*, tRNA nucleotidyltransferase is one of the best characterised (Williams and Schofield, 1977). Among eukaryotes, it has been characterised in yeast (Chen *et al.*, 1990), rabbit (Masiakowski and Deutscher, 1980), rat (Mukergi and Deutscher, 1972), wheat (Dullin *et al.*, 1975) and lupin (Cudny *et al.*, 1978-B).

In *E. coli*, tRNA nucleotidyltransferase analysed by SDS polyacrylamide gel

electrophoresis, had a molecular weight of 51 kDa, which was also in good agreement with the molecular weight of 53 kDa, obtained by column chromatography on a Sephadex G-100 column (Schofield and Williams, 1977) and is in reasonable agreement with the size predicted from the gene sequence. This also suggests that this protein exists as a monomer in the cell. In contrast to the *E. coli* tRNA nucleotidyltransferase, the yeast enzyme was slightly larger, 60 kDa, as estimated by SDS Polyacrylamide gel electrophoresis (Chen *et al.*, 1990). Again this is in good agreement with the size predicted from the amino acid sequence. The lupin tRNA nucleotidyltransferase was slightly smaller and estimated to be around 40 kDa (+ or - 5 kDa) by gel filtration on a Sephadex G-100 column (Cudny *et al.*, 1978-B). The pH optima for these enzymes from different sources were about the same: 8.5 - 9.25, 9.5 and 9.5 in *E. coli* (Schofield and Williams, 1977), yeast (Chen *et al.*, 1990) and lupin (Cudny *et al.*, 1978-B), respectively. The tRNA nucleotidyltransferase from wheat had two pH optima, 7.6 and 8.6, suggesting that there could be two isoforms of the same protein (Dullin *et al.*, 1975). It is possible that these two isoforms may represent cytoplasmic and mitochondrial or chloroplast forms of this enzyme. This prospect is particularly relevant with respect to this thesis since one of our long term goals is to determine whether or not there may be separate enzymes that function in each of these locations. Both wheat and lupin tRNA nucleotidyltransferases required Mg^{++} for optimal activity (Dullin *et al.*, 1975, Cudny *et al.*, 1978-B). KCl seemed to increase the activity of lupin tRNA nucleotidyltransferase at a concentration of 200 mM and maximum activity was observed at 43^{0}C (Cudny *et al.*,

1978-B).

Mukergi and Deutscher (1972) showed the presence of tRNA nucleotidyltransferase in rat mitochondria and its localisation in the mitochondrial matrix suggested that organelles in animal cells may also have their own tRNA processing machinery. They also showed that one third of the total tRNA nucleotidyltransferase in the cell was contained in the mitochondria. Although Mukerji and Deutscher (1972) suggested that this enzyme had a cytoplasmic location in rat liver cells and was absent from the nucleus, more recent work by Solari and Deutscher (1982) on the study of the subcellular localisation of the tRNA nucleotidyltransferase in *Xenopus laevis* revealed that this enzyme is present in both the cytoplasmic and nuclear compartments. They also showed that the enzyme readily leaks out of the nuclei during storage, *i.e.*, the enzyme probably leaked out of the nucleus during the experimental procedure and this may explain Mukerji and Deutscher's earlier observation. A nuclear localisation for tRNA nucleotidyltransferase has been confirmed in yeast because tRNAs which have not had their introns removed (Peebles *et al.*, 1979) and therefore cannot exit the nucleus have been shown to have a complete 3' terminal CCA.

5. THIS WORK

As discussed above, tRNA nucleotidyltransferase is an enzyme required for tRNA maturation in the nucleus, mitochondrion and chloroplast. Since previous studies (Chen *et al.*, 1992) have shown that the tRNA nucleotidyltransferase in yeast

is targeted to both the nucleus and the mitochondrion I was interested in finding out if a similar phenomenon existed in plants. I chose to study targeting in plants, because plants have a higher level of complexity for protein localisation than most other eukaryotes in that they have an additional organelle, the chloroplast, to which proteins must be targeted. My initial goal was to isolate a gene encoding tRNA nucleotidyltransferase in plants. With this gene in hand I hoped to identify targeting signals and to show how these signals function in protein targeting in plants. I chose tRNA nucleotidyltransferase because the literature contained protocols for purification of this enzyme from plants (Cudny *et al.*, 1978-A, Dullin *et al.*, 1975) and because this is an essential enzyme in the only other eukaryote (yeast) analysed. I used the purified lupin enzyme to obtain a partial amino acid sequence which I used to construct degenerate oligonucleotides for polymerase chain reaction to amplify a fragment of DNA which could later be used to select a full length cDNA clone that encodes the tRNA nucleotidyltransferase. With the purified protein in hand I was able to perform some basic characterisation of this enzyme.

II. MATERIALS AND METHODS

1. PROTEIN PURIFICATION

A) Preparation of crude extract

One kilogram of dry *Lupinus albus* seeds was ground manually in a meat grinder with a grating that had a pore size of 4 mm at 4^0C using 5 l of 50 mM acetate buffer (pH 6.0) (Cudny *et al.*, 1978-A). Acetate buffer was added periodically during the grinding process and the slurry was collected in a clean plastic tray. During the grinding process, the slurry in the acetate buffer was stirred from time to time using a glass rod. The total slurry was then divided into 6 X 1 l Nalgene centrifuge bottles and centrifuged at 2500 X g (2000 rpm) at 4^0C in an IEC centrifuge using the 276 rotor to sediment the larger seed pieces. The supernatant was filtered through two layers of cheesecloth and the resulting filtrate was divided into 8 X 500 ml Nalgene centrifuge bottles. These were centrifuged at 4424 X g in a Beckman centrifuge with the JA-10 rotor at 4^0C for twenty minutes to pellet particulate matter.

B) Ammonium sulphate fractionation

The supernatant was collected and ammonium sulphate added over a period of 30 minutes to 30% saturation. This was left undisturbed at 4^0C for one hour and

then transferred to 500 ml Nalgene centrifuge bottles which were centrifuged in the Beckman centrifuge with JA-10 rotor at 11 325 X g at 4°C for 20 minutes. The pellets were resuspended in a total of 150 ml MS buffer (50 mM Tris HCl [pH 8.0], 0.1 mM EDTA, 1mM ß-mercaptoethanol and 1 mM $MgCl_2$) (Cudny *et al.*, 1978-A) and stored at 4°C. Additional ammonium sulphate was added to the supernatant to bring it to 55% ammonium sulphate saturation. This was left undisturbed at 4°C for 1 h and centrifuged again as above. The 55% pellets were resuspended in a total of 150 ml MS buffer and stored on ice. An aliquot of the supernatant was saved to check for residual tRNA nucleotidyltransferase activity.

C) Dialysis

The 30% and 55% ammonium sulphate fractions resuspended in MS buffer as well as the 55% supernate were dialysed separately against 5 l of MS buffer for 4 hours at 4°C. Dialysis was repeated two additional times in the same volume of fresh MS buffer each time. Glycerol was added to a final concentration of 10 % to the dialysate which was stored at -76°C.

D) Protein concentration / BSA standard curve

Protein concentrations were determined following the procedure supplied with the BioRad protein assay kit (based on the standard Bradford assay) with minor modifications. The dye reagent concentrate was diluted 1:3 in distilled H_2O instead of a 1:4 dilution as recommended by the supplier since 1:3 gave reproducible

duplicate readings. To 800 μl of this dye reagent was added a total volume of 200 μl of sample + H_2O to make up a final volume of 1 ml. Absorbance was measured at 595 nm on a Perkin Elmer Cetus Lambda 3 spectrophotometer.

Each time a fresh batch of dye reagent was prepared from the concentrated stock, a standard curve was plotted with known concentrations (1, 2, 4, 6, 8, 12, 16, 20 and 24 μg) of bovine serum albumin. Protein concentrations in different samples were determined by linear regression analysis of a standard curve.

E) Measurement of tRNA nucleotidyltransferase activity

The standard procedure of Cudny *et al.* (1978-A) was used with several modifications. Wheat tRNA (type V, total wheat tRNA from Sigma) was vacuum dried in 1.5 ml Eppendorf tubes. To each tube containing 20 μg of dried tRNA was added glycine buffer (pH 9.0), $MgCl_2$, CTP, ATP and 1μl of a 1:10 dilution of [α^{32}P] ATP (10 μCi/μl), protein and sterile H_2O to 100 μl. Final concentrations of each were 0.1 M glycine, 10 mM $MgCl_2$, 0.2 mM CTP, 0.2 mM ATP, 0.0154 μM [α^{32}P] ATP. The amount of protein added varied from 2 μg to 20 μg depending on the degree of purification. This mixture was incubated at room temperature (21^{0}C-25^{0}C) for 20 minutes and the reaction stopped by adding 100 μl of cold (4^{0}C) 2N HCl. Samples were left on ice for 20 minutes and filtered through GF/F (Whatmann) glass fibre filters using a Millipore sampling manifold. Each filter was washed two times with 50 ml of 1N HCl to eliminate any unincorporated free [α^{32}P] ATP. A final wash with 20 ml of 99% ethanol was done to facilitate easy drying of the filters.

Filters were placed in a 37^{0}C incubator until they were completely dry (usually about 1h) and then placed in scintillation vials containing 5 ml scintillation fluid (Cytoscint from ICN). Counts were measured as cpm in duplicate for some and triplicate for others in a scintillation counter (LKB WALLAC - 1218 RACKBETA).

F) Column chromatography

The 30% - 55% ammonium sulphate fraction was used to further purify tRNA nucleotidyltransferase by column chromatography since most of the activity was seen in this fraction. The procedures of Cudny *et al.* (1978-A) for DEAE and hydroxylapatite chromatography and of Schofield and Williams (1977) for tRNA-Sepharose affinity chromatography were used with minor modifications. Columns were run with the Pharmacia peristaltic pump P-1 and fractions were collected using a Pharmacia Redifrac fraction collector.

i) DEAE Chromatography

A 2 X 40 cm Pharmacia column was packed with DEAE-Sepharose Fast Flow (pharmacia) at 10 ml/minute and equilibrated with 1 l MS buffer. Protein (7000 mg) was adsorbed and washing was continued with MS buffer for 9 h at a flow rate of 8 ml/min until the absorbance of the wash at 280 nm was below 0.1. Protein was eluted with 1 l MS buffer containing 60 mM KCl at a flow rate of 7 ml/minute. Fractions (7.5 ml) were collected and every third fraction was assayed for tRNA nucleotidyltransferase activity. Active fractions (40 - 65) were pooled and dialysed

three times against 4 l of 10 mM potassium phosphate buffer (pH 7.4) to be used in hydroxylapatite column chromatography.

ii) Hydroxylapatite chromatography

A 1.5 X 20 cm column (Pharmacia) was packed with 40 ml of hydroxylapatite resin (BioRad) at a flow rate of 0.8 ml/minute and equilibrated with 10 mM potassium phosphate buffer (pH 7.4). The dialysate from the DEAE column active fractions (460 ml) was adsorbed on the hydroxylapatite column and washed with 10 mM potassium phosphate buffer (pH 7.4). The protein retained on the column was eluted with a linear phosphate buffer gradient from 10 mM to 250 mM. Fractions (5 ml) were collected at a flow rate of 0.8 ml/minute. Every third fraction was assayed for tRNA nucleotidyltransferase activity. Active fractions were pooled and dialysed against 20 mM sodium phosphate (pH 6.0) to be used in tRNA-Sepharose affinity chromatography.

iii) tRNA-Sepharose affinity chromatography

The tRNA-Sepharose affinity resin was prepared as follows: 3 grams of CNBr activated Sepharose 4 B freeze dried powder (Pharmacia) were suspended in 10 ml of 1 mM HCl. The swollen gel was washed for 15 minutes with 1 mM HCl on a sintered glass filter. Total wheat tRNA type V (Sigma) served as the ligand. Lyophilised tRNA (35 mg) was dissolved in 15 ml coupling buffer (0.1 M sodium carbonate [pH 8.3], 0.5 M NaCl) and mixed with the gel in a 50 ml conical bottom

tube by end-over-end rotation at 10 rpm at room temperature for two hours. Excess ligand (uncoupled) was removed by centrifuging the ligand gel mixture using the JA-17 rotor at 137 X g for 5 minutes in a Beckman J2HS centrifuge. The supernatant was discarded and this procedure was repeated until the absorbance of the supernatant containing the ligand dropped to negligible at 260 nm. Following coupling, the remaining active groups were blocked with 0.1 M Tris-HCl buffer (pH 8.0). Finally, the gel coupled with tRNA was washed with three cycles of 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl followed by a wash with 0.1 M Tris buffer (pH 8.0) containing 0.5 M NaCl. This resin was packed in a 1 X 10 cm column at a flow rate of 0.7 ml/min.

The dialysate from the hydroxylapatite column (70.2 ml pooled from active fractions off 3 columns) was adsorbed on the tRNA-Sepharose affinity column at a flow rate of 1.2 ml/minute and washed with 20 mM sodium phosphate (pH 6.0) containing 10 mM $MgCl_2$, 15 mM 2-mercaptoethanol and 10% glycerol. Once protein was no longer detected in the sodium phosphate wash, washing was continued with 50 mM Tris HCl (pH 8.5) containing 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 20 % glycerol. After the protein concentration in this eluate dropped below detectable limits, tRNA nucleotidyltransferase was eluted with 50 mM Tris (pH 8.5) containing 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 200 mM NaCl, 1 mM EDTA and 20 % glycerol. Fractions were collected at a flow rate of 1.2 ml/minute and every second fraction was assayed for tRNA nucleotidyltransferase activity.

G) Concentration of tRNA nucleotidyltransferase from tRNA-Sepharose affinity column fractions

Fractions containing tRNA nucleotidyltransferase were pooled and transferred to dialysis tubing with a molecular weight cutoff of 7000 Daltons (Spectra/Por, 8-670A). The tubing filled with these fractions was overlaid with polyethylene glycol flakes (25000 Daltons molecular weight cutoff). Periodic changes in PEG (Fisher Scientific, CAS 37225-26-6), every half hour were done to facilitate the process of concentration at 4^0C.

H) SDS polyacrylamide gel electrophoresis

Mini SDS polyacrylamide gels consisting of a 12 % separating gel and a 4 % stacking gel were made according to the instructions accompanying the Biorad apparatus and gel preparation of Laemmli (1970). To analyze the level of purity of the protein of interest, fractions from different stages of purification were loaded and run at constant voltage (200) for 45 minutes.

Pooled concentrated tRNA nucleotidyltransferase from tRNA-Sepharose affinity fractions was loaded onto a 16 centimeter long SDS polyacrylamide gel and electrophoresed for 3 hours at constant voltage (200 V).

I) Staining

i) Coomassie blue staining

To visualise the proteins separated by SDS polyacrylamide gel electrophoresis, the gels were stained with Coomassie blue R-250 (0.1% in 40% methanol and 10% acetic acid) for thirty minutes in a clean glass tray. Destaining was carried out by placing the stained gel in 40% methanol, 10% acetic acid for one to three hours, changing the destaining solution every hour. Destaining sponges were used in the destaining process to enhance destaining.

ii) Silver staining

Silver staining was performed on SDS polyacrylamide gels according to the protocol of Silver Stain Plus kit of Biorad (Gottlieb and Chavka, 1987). In short, gels were fixed in fixative enhancer solution for 30 minutes in a clean glass dish. At the end of 30 minutes the fixative was decanted and the gels were rinsed twice in 200 ml deionised water, 10 minutes each time. Then the gels were transferred to a clean glass dish containing the staining and developing solution (contains silver complex solution, reduction moderator solution and image development solution). This was agitated gently. Gels were left in the staining and developing solution until desired intensity was reached and the reaction stopped by transferring the gels to a tray containing stop solution (5% acetic acid). Gels were left in stop solution for 10 minutes with gentle agitation.

J) Protein electroblotting for microsequencing

Blotting onto PVDF membrane (Biorad) was done using a Biorad Transblot cell as recomended by the manufacturer with several modifications. At the end of

electrophoresis, the gel was removed from the glass sandwich and soaked in transfer buffer (0.6 % Tris, 0.71 % glycine, 20 % methanol and 0.01 % SDS) for five minutes. During this time the PVDF was rinsed in HPLC grade 100% methanol and left in transfer buffer until used. Transfer was set up in the Transblot cell according to the manufacturer's instructions. Transfer was carried out for 2 hours at 0.5 amps using a Biorad 2000 power pack in a cold room (4^0C). To keep the transfer buffer as cold as possible the Transblot cell was immersed in an ice bath.

At the end of transfer, the PVDF membrane was washed with dH_2O for 1 minute. The washed blot was then stained with freshly prepared Ponceau stain (Sigma, 0.2 % Ponceau in 1 % acetic acid) for 1 minute, followed by destaining in 1 % acetic acid for ninety seconds, with gentle agitation. The band of interest from multiple lanes was cut out and these membrane pieces combined in one Eppendorf tube. Distilled water was added to this, vortexed for 15 seconds, and the water removed. The Eppendorf tube containing the bands exised from the blot was capped tightly and stored at -20^0C until microsequencing was performed. Microsequencing was carried out at the Harvard Microchem Facility, Harvard University, Cambridge, Massachusetts.

2. Characterisation of tRNA nucleotidyltransferase

A number of experiments were carried out to determine the optimum assay conditions for this enzyme.

A) Effect of glycine concentration

Standard assay conditions as mentioned in measurement of tRNA nucleotidyltransferase activity (section II. 1 E) were used except that the glycine concentration was altered to give 10 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM or 400 mM. The samples were incubated for 20 minutes and the amount of [α^{32}P] ATP incorporated measured as described previously.

B) Effect of pH

Using the standard assay conditions as mentioned above the effect of glycine buffers of differing pH was determined. Glycine at pH 7.5, 8, 8.5, 9, 9.5 and 10 was used. The effect of different pHs of TRIS and CAPS buffers were also tested using buffers of pH 7.5, 8, 8.5, 9, 9.5 and 10 for TRIS and 9, 9.5, 10, 10.5 and 11 for CAPS.

C) Effect of ATP and CTP concentrations

Under standard conditions as described above the following concentrations of ATP and CTP were used in the activity assay (0.01 mM, 0.1 mM, 0.2 mM, 1 mM and 10 mM). Reactions were stopped at 10, 20, 40 and 60 minute intervals.

D) Effect of temperature

The standard activity assay as described above was performed for tRNA nucleotidyltransferase activity at 10^{0}C, 20^{0}C, 30^{0}C, 37^{0}C, 45^{0}C and 65^{0}C to look at the

effect of temperature on the enzyme activity. Reactions were stopped at 0, 10, 20 and 40 minutes.

E) Effect of salts (ion requirements)

The effect on enzyme activity of both monovalent (KCl and NaCl) and divalent ($MgCl_2$ and $MnCl_2$) cations was determined in standard activity assays in which the salt concentrations were varied. KCl and NaCl at 100 mM, 200 mM, 400 mM and 600 mM were used as were $MgCl_2$ and $MnCl_2$ at 0.1 mM, 1 mM, 10 mM and 100 mM. Reactions were stopped at 0, 20, 40 and 60 minutes for KCl and NaCl and 0, 10, 20 and 60 minutes in the case of $MgCl_2$ and $MnCl_2$.

F) Effect of EDTA

The effect of EDTA on enzyme activity using 0.5 mM, 1 mM and 10 mM EDTA was examined in the standard activity assay as described above. Reactions were stopped at 0, 10, 20 and 60 minutes.

3. Isolation of a cDNA encoding lupin tRNA nucleotidyltransferase

The lupin cDNA library used in this study was made in the lambda zap vector supplied by Stratagene. The cDNA library prepared from total RNA extracted from the root tissue of *Lupinus albus* was kindly supplied by Dr. Sylvie Attucci.

A) Titering the lupin cDNA library

Luria broth (10 g bactotryptone, 10 g NaCl and 5 g yeast extract in 1 l H_2O) (5 ml) containing 0.2% maltose and 10 mM $MgSO_4$ was inoculated with 50 μl of an overnight culture of XL1 blue cells (SupE44 hsdR17 recA1 endA1 gyrA46 thi relA1 Lac$^-$F'[proAB$^+$ LacI^q LacZ Tn10(tet^r)]) (from Stratagene) and grown at 37^{0}C to an OD_{600} of 0.7. Cells were pelleted by centrifugation at 2204 X g using a JA-17 rotor in a Beckman J2HS centrifuge for 10 minutes at 4 C. The supernatant was discarded and the pellet resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5 by pipeting.

These cells were subsequently divided into 100 μl aliquots in 150 X 15 mm Fisher test tubes (sterile) and 10 μl of appropriate phage stock dilutions (10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}) in SM buffer (0.1 M NaCl, 0.01 M $MgSO_4$, 0.5 M Tris [pH 7.5], 1 X 10^{-4} g/ml gelatin) were added. The tubes were incubated at 37^{0}C for 25 minutes. At the end of incubation 3.5 ml of warm NZY top agar (same as NZY broth [5 g NaCl, 2 g $MgSO_4$, 5 g yeast, 10 g NZ-amine/l] except that it contains 0.7 % Bacto agar) was added to the test tubes containg the cells and the phage. This was mixed by vortexing gently and poured onto 85 mm NZY agar Petri plates. The plates were left at room temperature until the soft agar solidified and then were incubated at 37^{0}C for 8 hours. Plaques were counted manually.

B) *In vivo* excision

In vivo excision was done according to the procedure of Stratagene with minor modifications. In brief, 500 μl of an overnight culture of XL1 blue or SOL R cells were used to inoculate 50 ml Luria broth containing 0.2 % maltose, 10 mM $MgSO_4$

and 10 μg/ml tetracycline or Luria broth containing 50 μg/ml kanamycin, respectively. The cells were incubated at 37^{0}C to an OD_{600} of 1. Cells were pelleted by centrifugation for 10 minutes at 1239 X g at 4^{0}C using a JA-17 rotor. The pellets were resuspended in 10 mM $MgSO_4$ to an OD_{500} of 1.

XL1 blue cells (200 μl) from above were incubated for 15 minutes at 37^{0}C, in a 50 ml conical tube, with 10μl of 10^{-3} Lupin cDNA phage stock and 1μl of 2x 10^7 pfu/μl Exassist™ helper phage. At the end of 15 minutes 10 ml of sterile Luria broth were added and incubation continued for another 2.5 hours at 37^{0}C with gentle shaking. The tubes were then transferred to 70^{0}C for 20 minutes and centrifuged at 2500 X g for 10 minutes using a JA-17 rotor in a Beckman centrifuge to pellet the cell debris. The supernatant containing the Bluescript phagemid in filamentous phage particles was saved and stored at 4^{0}C.

The supernatant from above was mixed with 50 ml of SOL R cells prepared as described above and incubated at 37^{0}C for 15 minutes. This was then added to a flask containing 500 ml Luria broth and ampicillin (100μg/ml) and incubated at 37^{0}C overnight. Plasmid preparation was carried out according to the procedure of Applied biosystems (see section 3 E iii) except that the volume of the reagents used was scaled up to extract the Bluescript plasmids carrying the lupin cDNA library.

C) Polymerase chain reaction

Degenerate oligonucleotides CCA1:

(G/A)TC(N)GT(A/G)TT(A/G/T/)AT(A/G)TT(A/G)TA(A/G)AA where N represents

A, C, G and T in equimolar ratio at a single position (extending towards the 5' end of the gene) and CCA2: (TT(T/C)GGIACICCIGA(G/A)GA(G/A)GA(T/C) (extending towards the 3' end of the gene) were designed based on the predicted nucleotide sequence from the known amino acid sequence. These primers were used in the polymerase chain reaction (PCR) to isolate a tRNA nucleotidyltransferase cDNA fragment from the lupin cDNA library. The PCR reaction mix consisted of 420 pmoles of each primer, 1 μg of lupin plasmid cDNA library, 5 μg bovine serum albumin (Biocan), 10 mM dNTP's, 1 X Taq polymerase buffer and 1 μl Taq DNA polymerase (Biocan) in a final volume of 50 μl. This was overlaid with 75 μl mineral oil to avoid evaporation at high temperature.

The following control reactions included reagents as above, except that in

1) the cDNA was eliminated,

2) Bluescript plasmid alone instead of the cDNA library in Bluescript was used,

3) Primer CCA1 was omitted and

4) Primer CCA2 was omitted

The reaction was carried out in a Hybaid thermal cycler with a hot start at 94^{0}C for 4 minutes followed by 30 cycles of 94^{0}C/ 30 seconds, 50^{0}C/ 30 seconds and 72^{0}C/ 30 seconds. A final cycle at 72^{0}C for 5 minutes was done to complete the extensions. PCR products at the end of the program were stored at -20^{0}C.

D) Phenol freeze fracture

DNA fragments of interest from PCR were separated on a 1.5 % agarose

(ICN) gel cast in TEA (0.04 M Tris-acetate, 0.001 M EDTA) buffer. The phenol freeze fracture technique (Bewsey *et al.*, 1991) was used to isolate the appropriate fragments. In brief, an agarose block containing the DNA fragment of interest was crushed in a 1.5 ml Eppendorf tube. To this 500 μl of phenol was added, vortexed and placed in the -76^{0}C freezer for 30 minutes. Freezing was followed by thawing at 37^{0}C for 15 minutes. To the thawed sample an additional 400 μl of phenol was added, vortexed and placed again in the -76^{0}C freezer for 30 minutes. The sample was thawed at 37^{0}C for 15 minutes and 100 μl of TE (10 mM Tris-HCl [pH 8], 1 mM EDTA) was added. This was vortexed and centrifuged at 14000 X g for 10 minutes at room temperature in an Eppendorf centrifuge. The aqueous phase was collected and extracted with an equal volume of fresh phenol twice followed by two chloroform and one ether extraction. The ether layer was discarded and the DNA precipitated with one tenth volume 3 M sodium acetate and two volumes 99% ethanol. This was placed in -76^{0}C for 30 minutes and centrifuged at 14000 X g for 30 minutes at 4^{0}C in an Eppendorf centrifuge. The pellet was washed in 70 % ethanol, dried and resupended in an appropriate volume (20 μl) of sterile distilled water.

E) Cloning of the DNA fragment amplified by PCR

i) Ligations

The purified DNA fragment of interest was cloned into a PCR TA vector, by standard procedures (Sambrook *et al.* 1989). In brief, to 200-500 ng of vector and 100 ng of insert was added 1 unit ligase in a total volume of 50 μl 1 X ligase buffer

and incubated in a 14^{0}C incubator overnight.

ii) Bacterial transformation

The cloned fragment of interest was used to transform *E. coli* using the reagents and cells supplied in the PCR cloning kit of Stratagene. The heat shock procedure supplied by the manufacturer was followed without any modifications. In short, a sticky end ligation of PCR product and PCR TA vector supplied in the kit was followed by transformation of *E. coli* with one tenth the volume of the ligation mix (5μl). At the end of transformation, the cells were plated on LB agar containing 100 μg/ml ampicillin and 50 μg/ml kanamycin.

iii) Plasmid preparation

The transformants that resulted from the above transformation were inoculated into 5 ml terrific broth (12 g bacto-tryptone, 24 g bacto-yeast extract, 4 ml glycerol/l) containing 100 μg/ml ampicillin. These were incubated at 37^0 C overnight with shaking at 250 rpm. Plasmid was extracted using the modified alkaline lysis, PEG precipitation procedure of Applied Biosystems without any modifications. Plasmid DNA was resuspended in sterile distilled water and stored at -20^{0}C.

F) Characterisation of the PCR products

i) Restriction analysis on independent positives

Plasmids were digested with *Eco*RI to examine the size of the insert cloned

in the PCR TA vector. These were run for 2 hour on a 2% agarose gel electrophoresed at 80 volts.

ii) DNA sequencing

DNA sequencing was carried out according to the procedure of USB Sequenase™ version 2.0 with minor modifications. In short, to 4 μg of DNA in 8 μl of water, 4 μl of 2 M NaOH and 4 μl of 1 mM EDTA were added. The total volume was made up to 20 μl with sterile distilled water. The denaturing mix was vortexed, centrifuged briefly and left at room temperature for 10 minutes. To this, 10 μl of 7.5 M ammonium acetate and 90 μl of 99 % ethanol were added. This was placed in a -76^{0}C freezer for 20 minutes and DNA pelleted by centrifuging at 14 000 Xg for 25 minutes at 4^{0}C in an Eppendorf microcentrifuge. The DNA was washed with 70 % ethanol, dried and resuspended in 7 μl of sterile distilled water.

Annealing was done by adding 2 μl of Sequenase™ 5X reaction buffer and 1 μl of primer (reverse or -40 supplied in the kit) to the DNA, mixing and placing in a heat block set to 65^{0}C. At the end of two minutes the block was removed from the heating element and left at room temperature to cool slowly to 30^{0}C.

A labelling reaction mix was prepared according to the manufacturer's instructions and added to 10 μl of the annealed template primer. This was incubated at room temperature for 2 minutes and 3.5 μl aliquots were added to the termination mixes pre-warmed at 37^{0}C. Incubation was continued for 3 more minutes and the reaction stopped by adding 4 μl stop solution.

Samples were boiled for two minutes prior to loading on a 6% sequencing gel (7 M Urea, 5.7%Acrylamide, 0.3 % Bis-Acrylamide, 1 X TBE (0.045 M Tris-Borate, 0.001 M EDTA)). Sequencing gels (38 X 50 cm) were prerun at 1000 V, 1500 V and 2000 V for a total of 90 minutes. Samples were electrophoresed at a constant voltage of 2000 until the dye from the second loading had migrated 35 cm (when multiple loading were done) or 45 cm (for a single loading). At the end of the run gels were fixed for 20 minutes in 3 l of 10 % acetic acid, 10 % methanol. The gel was then transferred to Whatman # 1 filter paper, covered by Saran wrap and dried under vacuum for 60 minutes at 80^{0}C. Saranwrap was taken off the dried gel which was exposed to X ray film (Fuji) and left at room temperature for a minimum of 24 hours.

G) Preparation of probe to screen the library

The 75 bp PCR amplification product exised from the PCR TA vector was used as a probe in hybridisation. Fragment was excised with *Eco*RI, separated from the rest of the vector by electrophoresis on a 1.5 % agarose gel and purified by the phenol freeze fracture technique (see section II 3 D). The fragment was labelled with [α^{32}P] dCTP using the random priming kit of United States Biochemical following the protocol supplied by the manufacturers. Briefly, 1 μl of DNA (approximately 25 ng) was added to 8 μl of sterile water and boiled for 10 minutes. The DNA was immediately cooled on ice and 1 μl dATP mix, 1 μl dGTP mix, 1 μl dTTP mix, 2 μl reaction mix and 5 μl [α^{32}P] dCTP (10 mCi/ml, 3000 Ci/mmole) were

added and mixed well. Finally, 1 μl of Klenow enzyme was added and the reaction was incubated at 37^{0}C for a minimum of 4 hours.

To purify the probe generated by random priming, 10 μl of blue dextran dye and 20 μl TE were added to the labelling reaction mix. This was passed through a Sephadex G50 column and approximately 500 μl of the blue fraction was collected. To check the percent incorporation of [α^{32}P] dCTP scintillation counts were taken for 1 μl of the labelling reaction mix before and after passing it through the column. An incorporation of 15 - 20 % was considered good.

H) Screening of lupin cDNA library

i) Phage infection

A single colony of XL1 Blue cells was inoculated into 5 ml of NZY medium and grown overnight at 37^{0}C. An aliquot of this overnight culture (500 μl) was used to inoculate 50 ml NZY medium containing 0.2 % maltose and 10 mM $MgSO_4$ in a sterile 500 ml Erlenmeyer flask. This was incubated with shaking at 37^{0}C until an OD_{600} of 0.6 was reached. Cells were pelleted by centrifuging at 1239 X g in a Beckman J2HS centrifuge using a JA14 rotor at 4^{0}C and resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.6.

The phage stock lupin cDNA library was diluted in SM buffer by a factor of one thousand and 6 μl was added to 600 μl of XL1 Blue cells in a sterile 10 ml tube. The phage and the cells were incubated at 37^{0}C for 25 minutes with gentle shaking (80 rpm). At the end of incubation, 7.5 ml warm NZY top agar was added to each

tube, vortexed gently and poured onto a 137 mm diameter NZY agar plate. Plates were left at room temperature until the soft agar solidified and then incubated at 37^0C overnight. This resulted in approximately 50 000 plaques/plate. At the end of incubation plates were left at 4^0C for at least 4 hours before proceeding with plaque lifts.

ii) Plaque lifts

Transfer of plaques from the agar plates to nylon membranes was carried out according to the instructions supplied by Amersham. Nylon membranes (Hybond N) were placed on the surface of the agar plates containing plaques in such a way so as to avoid trapping air bubbles between the membrane and the plate. Each plate was lifted in duplicate with the first filter in contact with the plate for 2 minutes and the second for 4 minutes. To orient the membranes, holes were punched through the duplicate membranes from the same plate at the same points on the agar plate with a red hot needle. After removing the membranes from the plates they were placed plaque side up on filter paper saturated with denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes. Excess solution was removed by blotting the membrane on a clean piece of Whatman # 1 filter paper. These filters were then placed on neutralisation solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.2], 0.001 M EDTA) for 3 minutes followed by another 3 minutes on fresh neutralisation solution. The membrane was then washed gently in 2X SSC briefly. Wet membranes were air dried for an hour at room temperature by placing them on Whatman # 1 paper.

Finally, they were baked in a vacuum oven for 2 hours at 80^{0}C wrapped in tinfoil between Whatman # 1 paper.

iii) Hybridisation

Ten membranes were placed in one 4 X 30 cm Hybaid™ hybridisation bottle with nylon mesh between each 2 filters. Membranes were prehybridised for one hour at 58^{0}C in 50 ml prehybridisation solution: 5 X SSPE (20 X = 3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA [pH 7.2]), 5 X Denhardts (100 X = 2% BSA, 2% Ficoll, 2% PVP), 0.5% SDS and 0.1 mg/ml denatured herring sperm DNA. Following prehybridisation the labelled probe which had been heated to boiling for 10 min and cooled on ice was added to the prehybridisation solution. Hybridisation was carried out at 58^{0}C overnight at maximum rotation in a Hybaid hybridisation oven.

iv) Washing

At the end of hybridisation, membranes were washed in 2X SSPE, 0.1% SDS for 15 minutes at room temperature. This was followed by two more washes in 1X SSPE, 0.1 % SDS at 58^{0}C for 1 hour each wash. Excess buffer was removed from the membranes and duplicates were wrapped together in Saran wrap. These were exposed to X-ray film with intensifying screens for 36 hours at -76^{0}C.

v) Developing

Films were developed in Kodak developer for 2 minutes, washed in water briefly and fixed in Kodak fixative for 2 minutes. Fixed films were thoroughly washed in water and air dried before examining.

vi) Plaque purification

Duplicate positive signals on the autoradiographs were aligned on the plate and plugs were pulled out using the thick end of a Pasteur pipette. Plugs were placed in 500 μl SM buffer with 3 drops of chloroform. This was vortexed and placed at room temperature for 3 hours for the phage to diffuse out of the agar. These were stored at 4^0C.

Plaques showing positive hybridisation were isolated for further rounds of purification. The region of the agar plate containing the plaques showing positive hybridisation was removed as an agar plug using a 1 mm diameter Pasteur pipette. The smallest diameter pipette possible was used to reduce the amount of contaminating plaque particles that would be isolated. Plaque from the plug was diluted to approximately 100 plaques/plate and hybridisation was carried out as explained above. Positive plugs were placed in 500 μl of SM buffer containing 3 drops of chloroform at room temperature for three hours and stored at 4^0C until used.

Regions of the agar plate showing positive hybridisation were isolated as described above and diluted in SM buffer to approximately 50 plaques / plate. Hybridisation was repeated as described earlier. A second round of purification

served to confirm the purity of the phage particles isolated previously as close to 100 % of the plaques in this secondary screen showed positive hybridisation. Positive plugs were pulled again and stored in SM buffer until *in vivo* excision could be carried out on the independant positives.

4. Characterisation of Lupin cDNA clones

A) Restriction analysis

To find out the approximate sizes of the cDNA inserts of the different positives they were subjected to restriction analysis with *Eco*RI and *Xho* I restriction enzymes. The products of restriction digestion were examined on a 1.5 % agarose gel. The largest clone was picked from the positives and used to produce deletions for sequence analysis.

B) Production of deletions

Deletions were produced using the Erase-a-Base™ kit of Promega following the instructions supplied by the manufacturer. Briefly, prior to starting deletions, 5 μg DNA was digested with *Sac*I or *Not*I first for 3 hours and then with *Kpn*I or *Xho*I to protect the primer binding sites (for sequencing) in the vector and to aid the Exo III to erase the appropriate DNA strand. The digested DNA was extracted twice with phenol and once with chloroform. This was precipitated with 3 M sodium acetate and 99% ethanol, centrifuged at 14 000 X g for 25 minutes, the pellet washed in 70% ethanol, dried and resuspended in 0.03 ml ExoIII buffer. This was left at

37^{0}C and 500 units of Exo III were added and mixed rapidly. Aliquots (2.5 μl) were removed at 30, 60, 90, 120, 150, 180, 210, 240, 270 and 300 seconds for each strand, added to S1 nuclease mix (7.5 μl) and incubated at room temperature for 30 minutes. The reaction was stopped by adding 1 μl of S1 stop buffer to each tube. S1 was inactivated by heating the reactions at 70^{0}C for 10 minutes. At this point 2 μl aliquots from each time point were checked by agarose gel electrophoresis for the extent of deletions. Reactions were transferred to 37^{0}C after heat inactivation of S1 and 1 μl of Klenow mix (30 μl of Klenow buffer and 3-5 u of Klenow DNA polymerase) added to each sample. This was incubated for 3 minutes and then for an additional 5 minutes on addition of dNTP mix. Ligation mix (40 μl) was added to each sample and incubated at 4^{0}C overnight.

One tenth the volume of total ligation mix was used to transform *E. coli* and the resulting transformants were patched to obtain good quantities of cells on a plate to perform a rapid screening of the same. In brief, the rapid screening involved smearing cells on the bottom of a clean Eppendorf tube and adding 50μl of 10 mM EDTA. This was mixed by vortexing and 50 μl of freshly prepared cracking buffer (2 ml 5M NaOH, 0.5 ml 10% SDS, 10 g sucrose per 50 ml) was added and vortexed. This was incubated at room temperature for 5 minutes. After 5 minutes 1.5 μl of 4 M KCl and 0.5 μl of 0.4% bromophenol blue were added and vortexed. This mixture was left on ice for 5 minutes followed by centrifugation at 11 000 rpm in an Eppendorf centrifuge for 3 minutes at 4^{0}C. An aliquot of the above preparation (25 μl) was loaded onto a 0.7% TEA agarose gel and electrophoresed for 2 hours at 80

volts. Plasmids carrying inserts covering the entire length of the cDNA clone in both directions were selected from the gels, the plasmids isolated (see section II 3 E iii) and sequenced (see section II 3 F ii).

III. RESULTS

1) PURIFICATION OF tRNA NUCLEOTIDYLTRANSFERASE

A) Ammonium sulphate fractionation

Very little of the tRNA nucleotidyltransferase activity was found in the 30 % ammonium sulphate fraction (Table 1) while the majority of the activity was found in the 30% - 55% ammonium sulphate fraction (Table 1). This fraction gave a total protein yield of approximately 6000 mg/1 kg of dry *Lupinus albus* seeds with a specific activity of 3 - 4 X 10^4 units/mg of protein in 2 separate experiments (Table 1) and was subsequently used in further purification steps.

B) DEAE column chromatography

As the next step in purification DEAE column chromatography was chosen as Cudny *et al.* (1978 A) had shown a 6.5 fold purification using this procedure. Based on the increase in specific activity a 7.5 - 9 fold purification from the 30 - 55% ammonium sulphate fraction was shown (Table 1). The proteins that did not bind to the resin were eluted in the MS buffer wash and were monitored by checking the presence of protein in every third fraction of the MS buffer wash spectrophotometrically at 280 nm. These fractions were not assayed for tRNA nucleotidyltransferase activity. The column was washed with MS buffer until the absorbance of these fractions was below an $O.D._{280}$ of 0.1 the MS buffer

TABLE 1

PURIFICATION OF LUPIN tRNA NUCLEOTIDYLTRANSFERASE

Purification Step	Crude		30%		55%		DEAE		HA		tRNA	
	1	2	1	2	1	2	1	2	1	2	1	2
Volume (ml)	3900	-	100	-	150	300	225	460	120	260	-	2.5
Protein (mg)	12948	-	2007	-	6600	12000	346.5	748	27.5	70.2	-	0.0825
Total activity (cpm)X 10^7	24	-	0.3	-	21.9	48	10	22	11	36	-	1.84
Specific activity (cpm/mg) X10^5	0 18	-	0 015	-	0.33	0.4	2.9	3	40	51	-	2230
Fold purification	1	-	-	-	1.8	1	16	7.5	222	128	-	5575
% Recovery	100	-	< 1	-	91	100	42	46	46	75	-	3.8

(-) indicates that these fractions were not assayed

Trial 1 is with 1 Kg of lupin seeds and Trial 2 is with 2 Kg of lupin seeds.

containing 60 mM KCl was started. The protein peak eluted with MS buffer containing 60 mM KCl paralleled the activity peak of tRNA nucleotidyltransferase (Fig. 2). The specific activity achieved by DEAE chromatography was about 30 X 10^5 (Table 1). DEAE chromatography also helped to eliminate endogenous tRNA which was confirmed by activity assays in which active fractions from the DEAE column or from the 30-55 % ammonium sulphate cut were added to the reactions in the absence of added tRNA. In this case no activity was seen in the DEAE column fraction, although activity was present in the 30-55 % fraction, presumably due to the presence of endogenous tRNA (data not shown).

C) Hydroxylapatite column chromatography

Hydroxylapatite column chromatography resulted in an approximately 128 fold purification of the tRNA nucleotidyltransferase over the levels in the 30-55% ammonium sulphate fraction (Table 1). After the tRNA nucleotidyltransferase had been adsorbed to the hydroxylapatite resin, it was eluted between 85 mM and 100 mM potassium phosphate buffer (Fig. 3). A sizeable quantity of the protein was present in the void volume but was devoid of any tRNA nucleotidyltransferase activity. The activity peak was found at the tailing end of the protein peak eluting in the potassium phosphate gradient, such that a majority of the protein was eliminated in this step. The total protein pooled from active fractions at the end of two hydroxylapatite columns with maximum tRNA

FIGURE 2 DEAE CHROMATOGRAPHY

(ELUTION AFTER ADDITION OF 60 mM KCl)

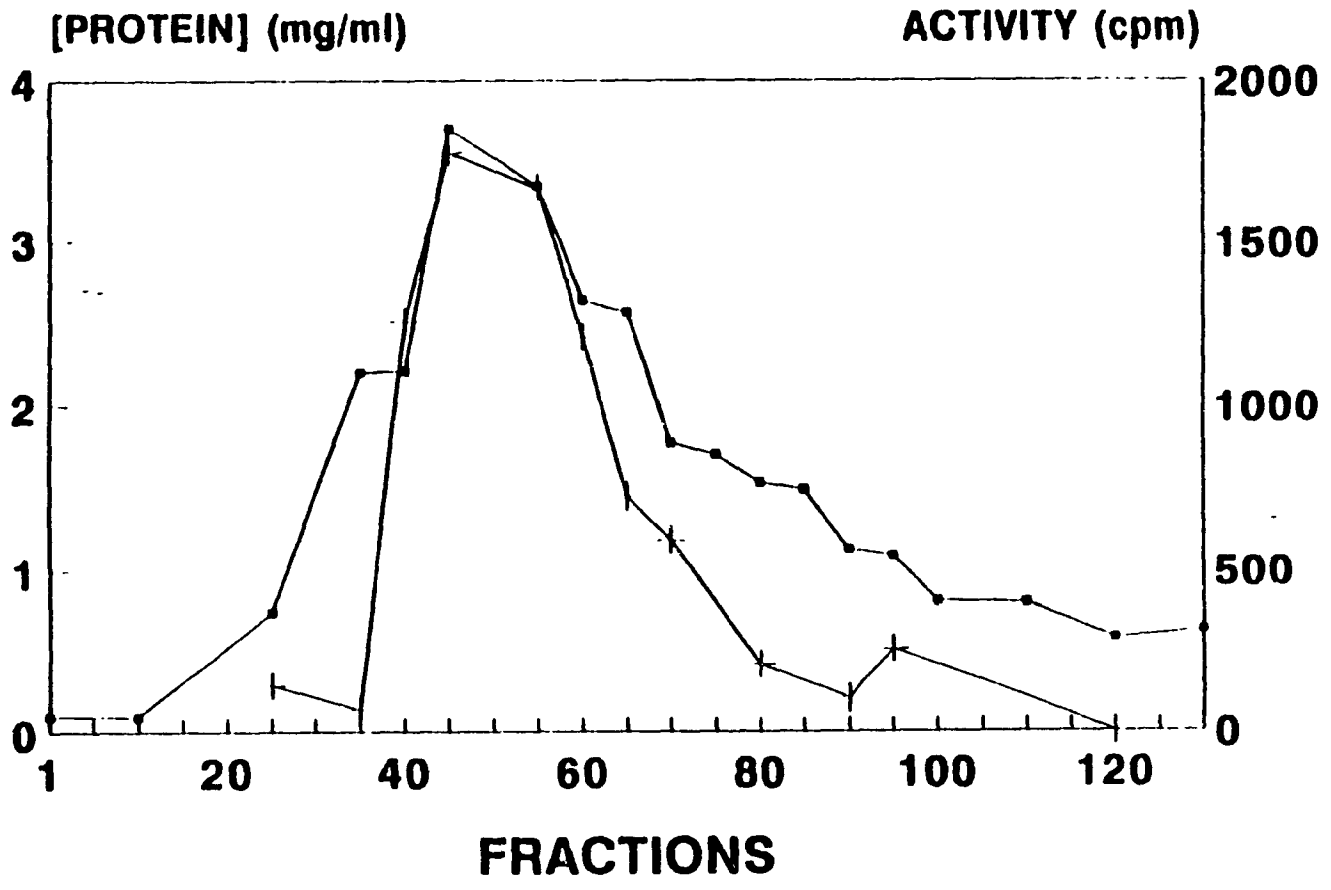
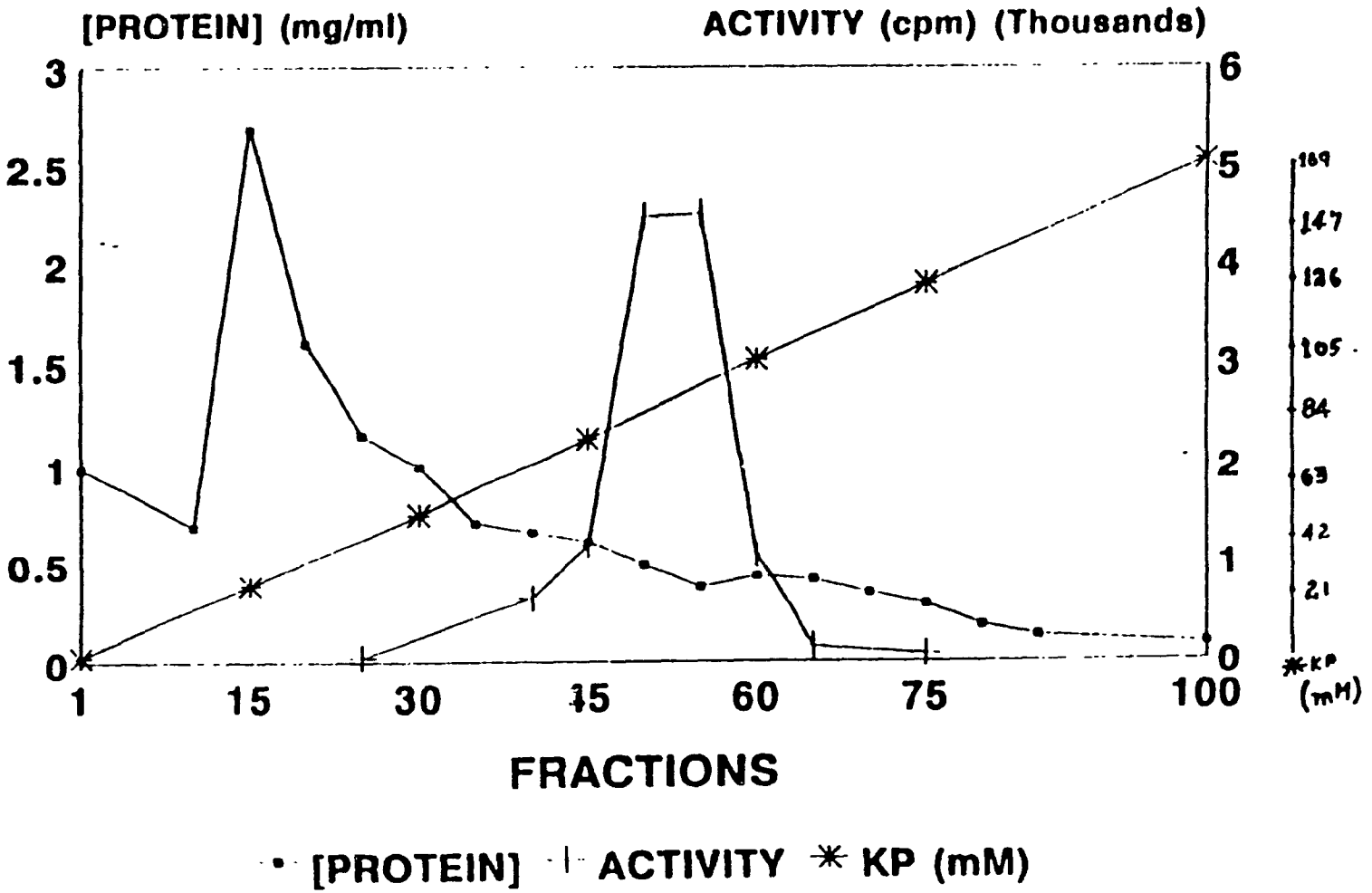


FIGURE 3

HYDROXYLAPATITE CHROMATOGRAPHY

(ELUTION BY POTASSIUM PHOSPHATE GRADIENT IS SHOWN)



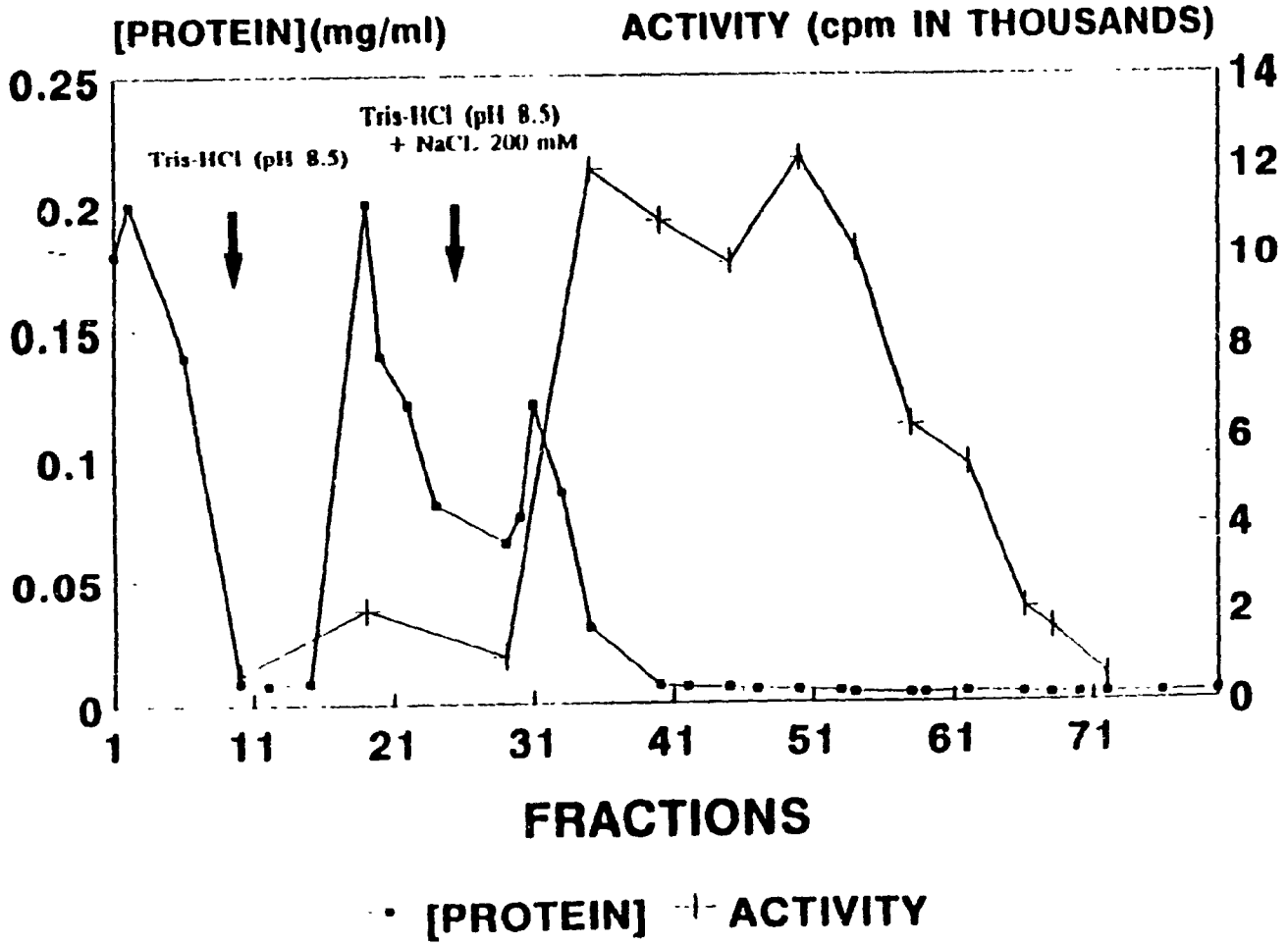
nucleotidyltransferase activity was 70.2 mg. The specific activity of active fractions pooled from 2 hydroxylapatite columns was 511 X 10^4 cpm/mg of protein (Table 1).

D) tRNA affinity column chromatography

The eluate from two hydroxylapatite columns was combined and loaded on to a tRNA-Sepharose affinity column. This column was used as a final step in purification because it is specific for tRNA modifying enzymes. Though many different tRNA modifying enzymes can bind tRNA, they can be purified based on their affinity for tRNAs. This final purification by tRNA- Sepharose affinity chromatography resulted in a 5575 fold purification of tRNA nucleotidyltransferase activity over that in the 30-55% ammonium sulphate fraction (Table 1). The majority of the protein from the active HA fraction were not retained on the tRNA-Sepharose column. Approximately 1.2 mg of the protein which bound to the tRNA Sepharose resin was eluted in the sodium phosphate (pH 6) wash. By changing the pH and the buffer to 50 mM Tris-HCl (pH 8.5) 3 mg more protein were eluted (Fig. 4). Finally the remaining proteins including the tRNA nucleotidyltransferase were eluted with 50 mM Tris-HCl buffer (pH 8.5) with 200 mM NaCl and 1.0 mM EDTA. The fractions (41 - 51; 1ml/fraction) containing tRNA nucleotidyltransferase with maximum activity had protein concentrations below 0.01 mg/ml (Fig. 4).

Because the protein concentration was below the detection limit of our

FIGURE 4 tRNA SEPHAROSE CHROMATOGRAPHY



assay an aliquot (150 μl) of every active fraction from the tRNA affinity column was separated on an SDS polyacrylamide gel and stained with silver stain. After checking the level of purity in this way, fractions (41-51) with the most pure tRNA nucleotidyltransferase were pooled and concentrated four fold (from 10 ml to 2.5 ml) using polyethylene glycol to give a specific activity of 22 300 X 10^4 cpm/mg of protein (Table 1).

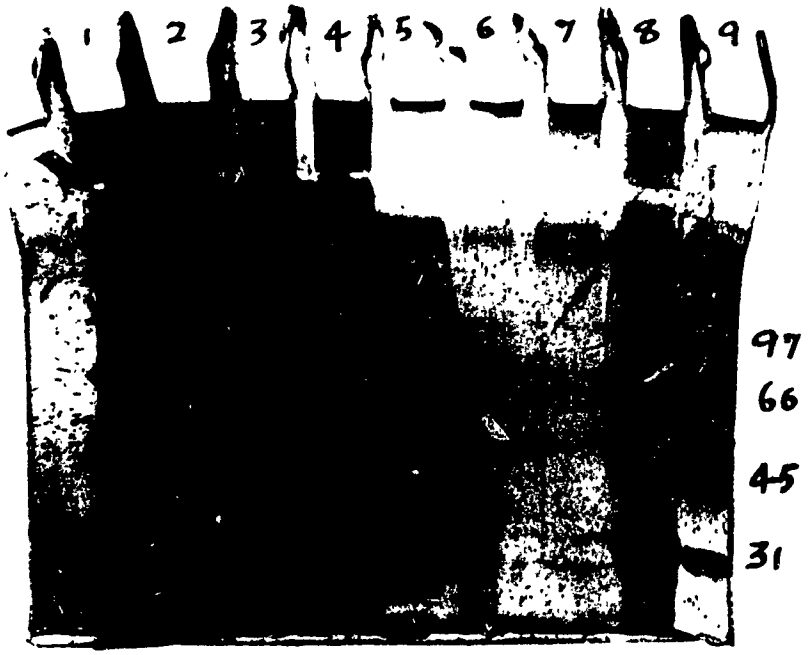
An SDS polyacrylamide gel (Fig. 5) shows the extent of purification at each stage of column chromatography. Lanes 1, 8 and 9 contain low molecular weight markers. Lane 2 to 7 show the enrichment for the tRNA nucleotidyltransferase protein through various purification procedures.

2) CHARACTERISATION OF tRNA NUCLEOTIDYLTRANSFERASE

The raw data for the characterisation experiments are listed as appendices. The tremendous variability in the apparent amount of [α^{32}P] ATP incorporated in the different experiments, relates directly to the age of the isotope used. Data presented in the histograms are averages of duplicate or triplicate values. Error bars represent the average error at each point.

Figure 5

Silver stained SDS-polyacrylamide gel showing purification of the lupin tRNA nucleotidyltransferase.



Lanes 1, 8 and 9 - molecular weight standards

Lane 2 - crude extract (20 μg)

Lane 3 - 30 % ammonium sulphate fraction (26 μg)

Lane 4 - 55 % ammonium sulphate active fraction (26 μg)

Lane 5 - DEAE-Sepharose active fraction (18 μg)

Lane 6 - Hydroxylapatite active fraction (14 μg)

Lane 7 - tRNA-Sepharose active fraction (4 μg)

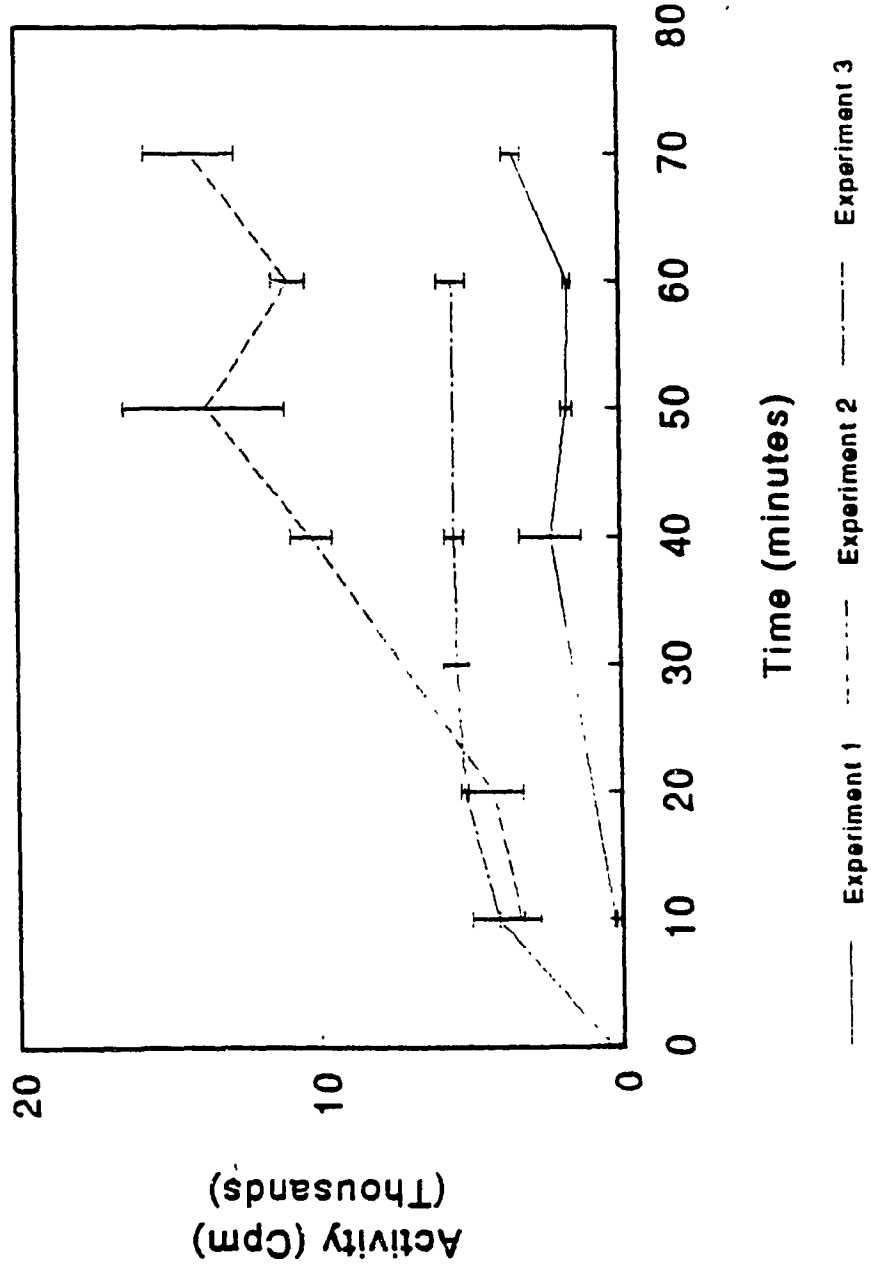
A) Time course

In a final volume of 100 μl each reaction contained 100 mM glycine (pH 9), 10 mM $MgCl_2$, 0.2 mM CTP, 0.2 mM ATP, 1μl of 1 in 10 diluted [α^{32}P] ATP and 20 μg of wheat tRNA. To this was added 1 μl of tRNA nucleotidyltransferase diluted 1 in 10 from a stock of 0.0825 mg/ml. The final concentration of the enzyme in a 100 μl reaction mix was 0.0825 μg/ml. This was incubated at room temperature (21^{0}C). The experiment was done three times, twice in duplicate and a third time in triplicate. These data are listed in Appendix A. The data showed some degree of variability between experiments, however, from these data (Fig. 6) one can suggest that a 20 minute time point is near the linear portion of the graph if not part of it. Because the 20 minute time point was near the end of the linear portion or at the begining of the plateau it was chosen as the time of incubation for further studies.

B) Effect of differing concentrations of glycine buffer (pH 9) on the activity of tRNA nucleotidyltransferase

Standard conditions were used except that Glycine (pH 9) at 10 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM and 400 mM was tested in the above reaction. Reactions were stopped with 100 μl cold 1N HCl at the end of 30 minutes incubation at room temperature (21^{0}C). The experiment was done twice with each reaction in duplicate (see Appendix B). Activity of tRNA nucleotidyltransferase increased in increasing glycine concentrations

FIGURE 6


Effect of incubation time on the incorporation of radiolabelled ATP
Activity (Cpm) (Thousands)
20
10
0
0
10
20
30
40
50
60
70
80
Time (minutes)
Experiment 1
Experiment 2
Experiment 3

up to 100 mM after which point increasing glycine concentrations did not seem to have a significant effect on the activity of tRNA nucleotidyltransferase (Fig. 7).

C) pH optimum

Standard conditions were used except Glycine (100 mM) at pH 7.5, 8, 8.5, 9, 9.5 and 10 were checked. The experiment was done twice with each reaction in duplicate the first time and triplicate the second time (see Appendix C). A gradual increase in the activity of the tRNA nucleotidyltransferase is apparent as the pH rises from 7.5 to 9.0 (Fig. 8). The activity of the tRNA nucleotidyltransferase appears to be at a maximum around pH 9 - 9.5 and drops gradually by pH 10 suggesting that the pH optimum for this enzyme is approximately 9 - 9.5. In the case of Tris-HCl buffer the optimum pH was seen to be around 8 - 8.5 while CAPS buffer had a very high pH optimum of 10 (Data not shown).

D) Effect of differing concentrations of cold ATP and CTP on the activity of tRNA nucleotidyltransferase

Standard conditions were used except that the effects of cold ATP and CTP at 0 mM, 0.01 mM, 0.1 mM, 0.2 mM, 1 mM and 10 mM were examined. Experiments were done three times with the reactions carried out in duplicate twice and in triplicate once (Appendix D & E). Figures 9 and 10 show the

FIGURE 7

EFFECT OF [GLYCINE] ON THE INCORPORATION OF RADIOLABELLED ATP

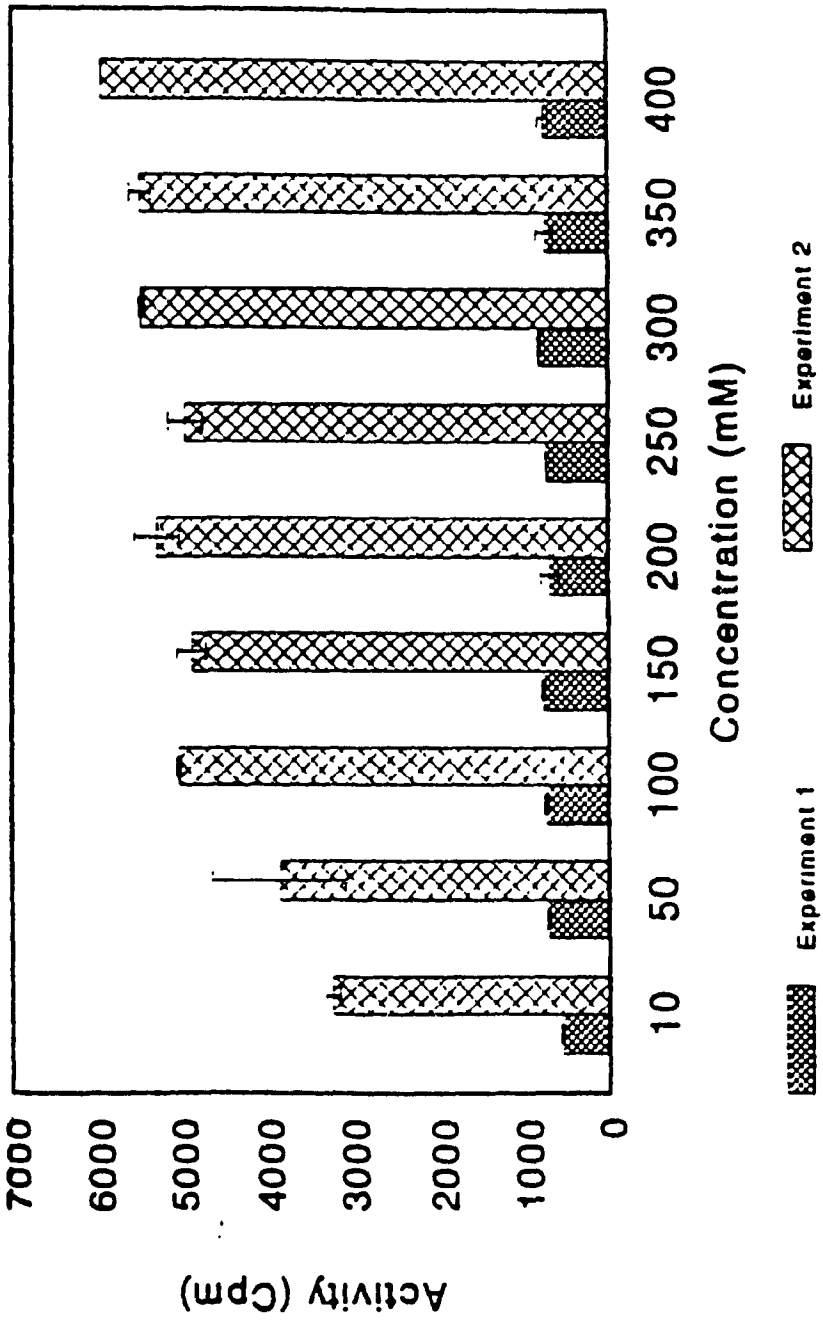


FIGURE 8

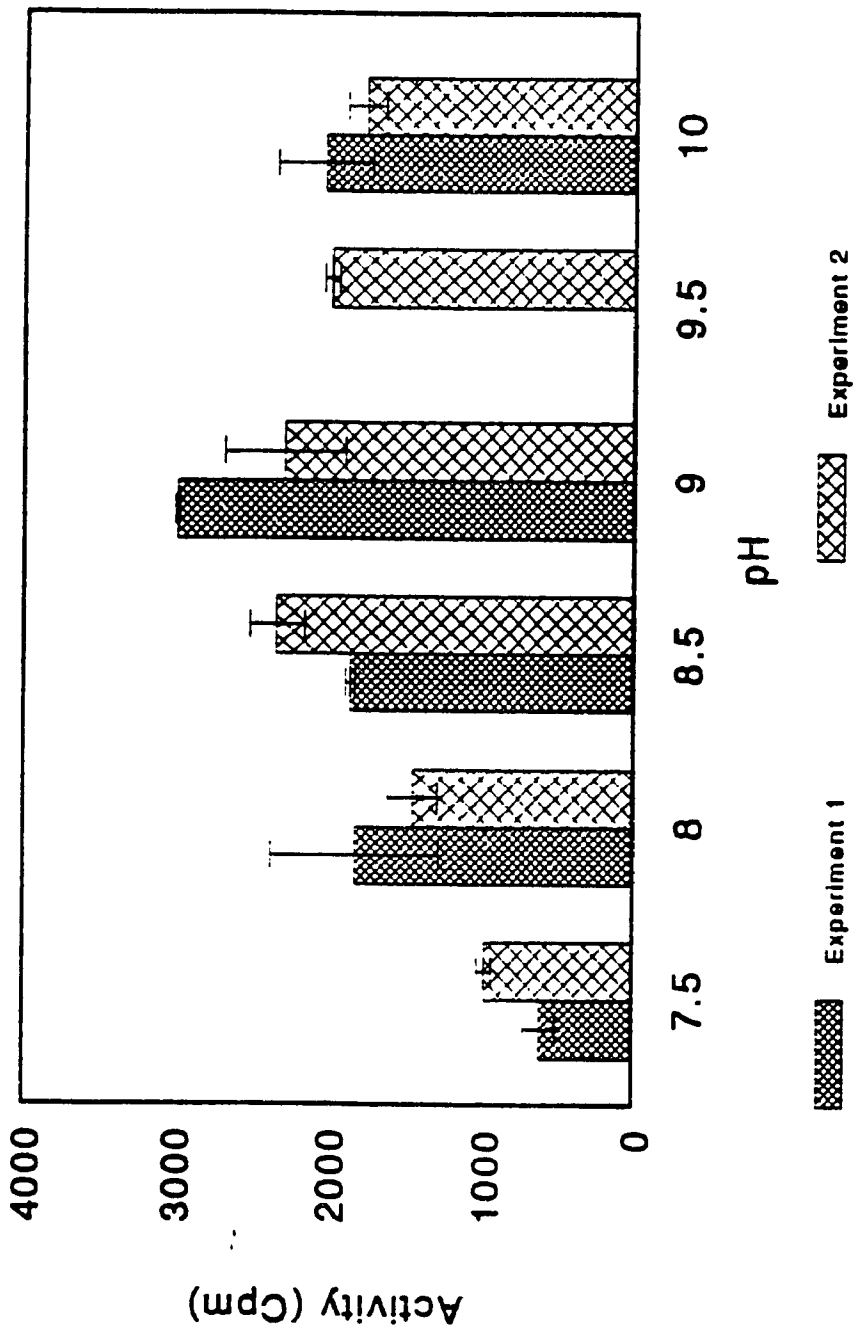

Effect of pH of glycine buffer on the incorporation of radiolabelled ATP
Activity (Cpm)
4000
3000
2000
1000
0
7.5
8
8.5
9
9.5
10
pH
Experiment 1
Experiment 2

FIGURE 9

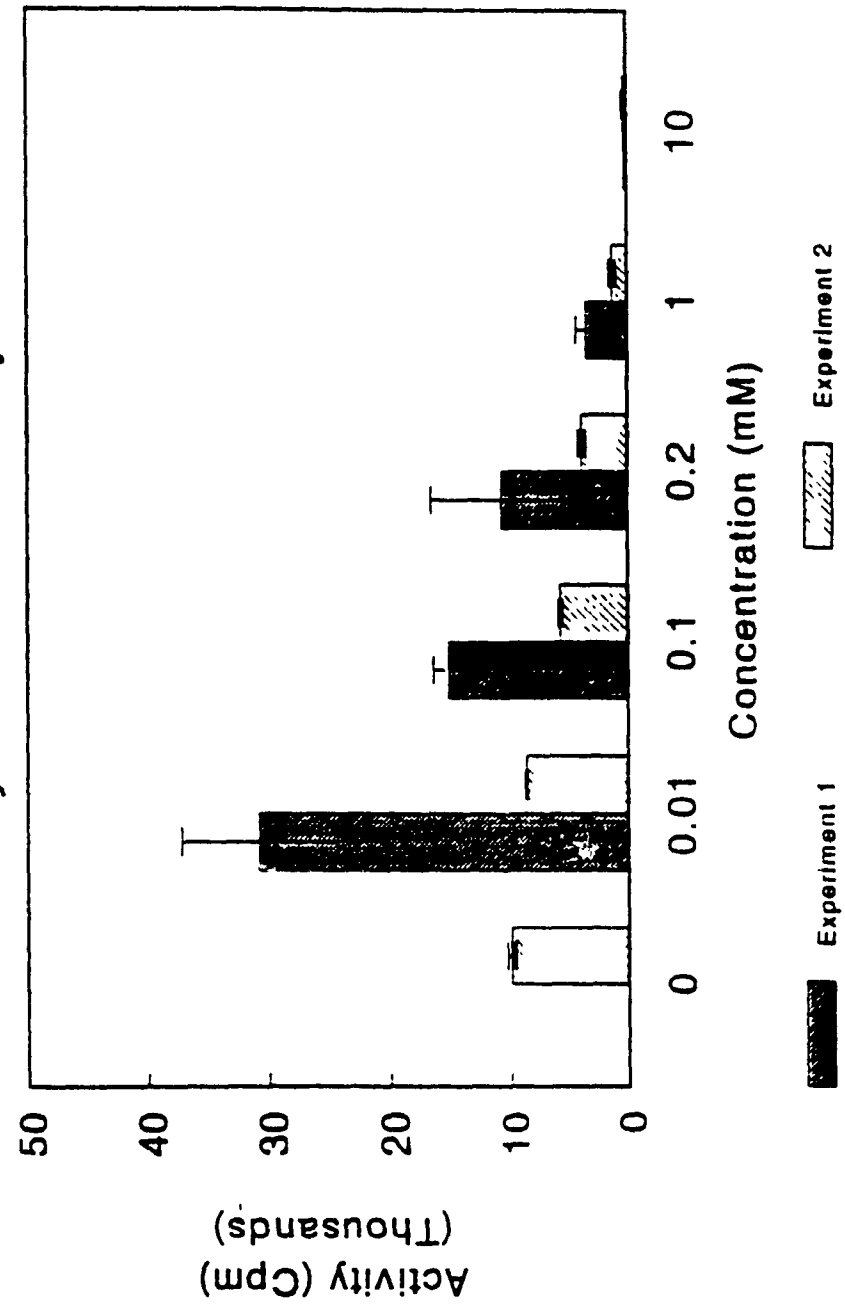
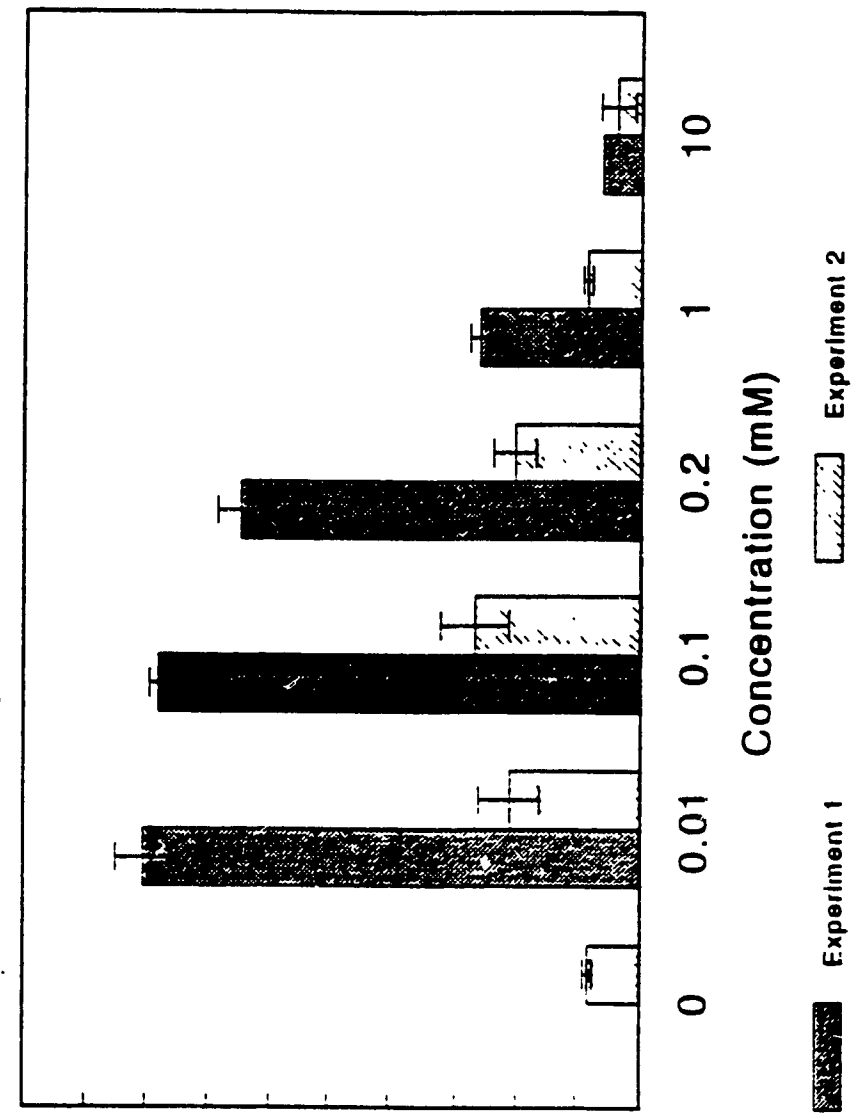

Effect of [ATP] on the incorporation of 32P ATP by tRNA nucleotidyltransferase
Activity (Cpm)
(Thousands)
50
40
30
20
10
0
0
0.01
0.1
0.2
1
10
Concentration (mM)
Experiment 1
Experiment 2

FIGURE 10


Effect of [CTP] on the incorporation of alpha 32P ATP
Activity (Cpm)
(Thousands)
10
9
8
7
6
5
4
3
2
1
0
0
0.01
0.1
0.2
1
10
Concentration (mM)
Experiment 1
Experiment 2

incorporation of [α^{32}P] ATP for differing concentrations of ATP and CTP, respectively. The maximum incorporation of [α^{32}P] ATP was seen when no non-radioactive ATP was used (Fig. 9). Non-radioactive ATP at 0.01 mM caused only a minimal drop in the incorporation of [α^{32}P] ATP, however, 10 mM non-radioactive ATP almost completely eliminated the incorporation of [α^{32}P] ATP indicating that it completely diluted out the radioactive ATP.

The level of [α^{32}P] ATP incorporation at different CTP concentrations after 20 minutes is shown in Fig. 10. There is a gradual increase in the incorporation of [α^{32}P] ATP from 0 mM CTP to approximately 0.1 mM CTP, after which there is a decrease in incorporation.

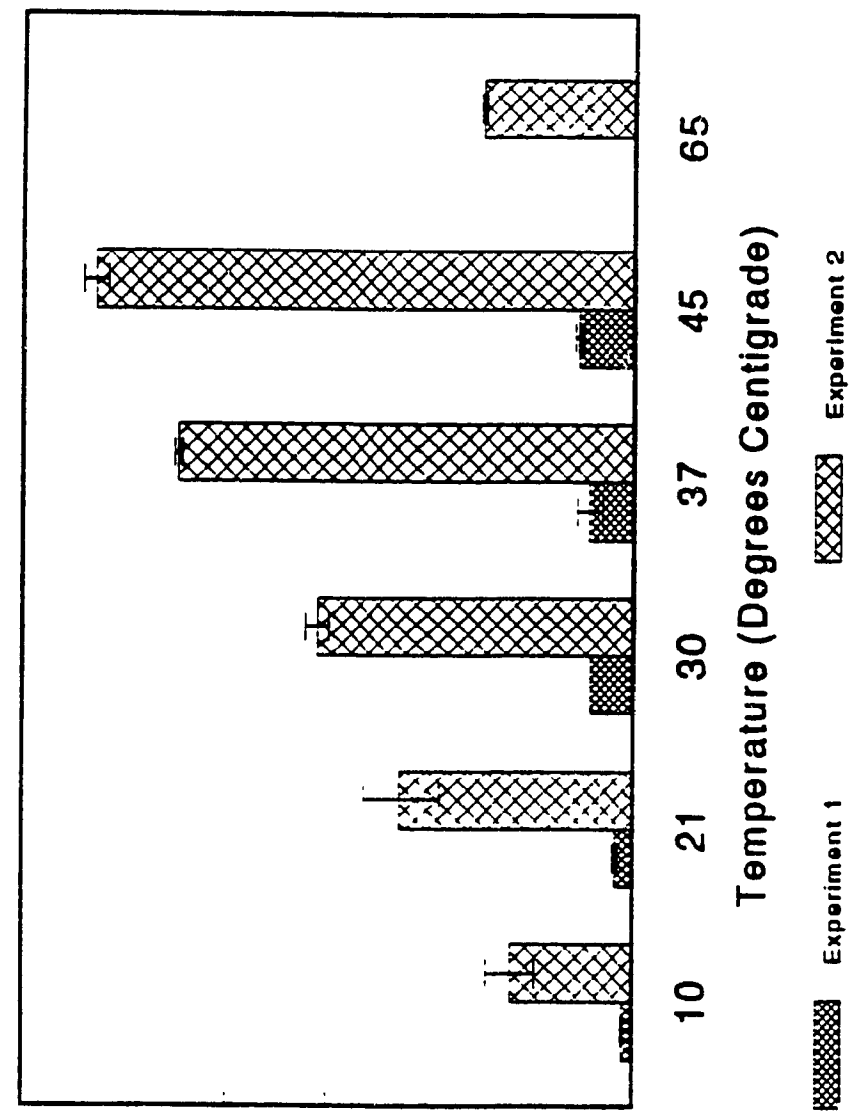
E) Effect of temperature

Standard conditions were used except reactions were incubated at 10^{0}C, 21^{0}C, 30^{0}C, 37^{0}C, 45^{0}C and 65^{0}C. Figure 11 shows activity at different temperatures for the 20 minute time point from each. Each experiment was performed twice with the reactions performed in duplicate (Appendix F). The level of tRNA nucleotidyltransferase activity increased to 45^{0}C with a rapid decrease at 65^{0}C.

F) Requirements for metal ions

Using standard conditions, different concentrations (0 mM, 100 mM, 200 mM, 400 mM and 600 mm) of NaCl and KCl were checked (Appendices G & H,

FIGURE 11


Effect of temperature on the activity of tRNA nucleotidyltransferase
Activity (Cpm)
6000
5000
4000
3000
2000
1000
0
10
21
30
37
45
65
Temperature (Degrees Centigrade)
Experiment 1
Experiment 2

respectively). The effects of various concentrations of $MgCl_2$ and $MnCl_2$ (0 mM, 0.1 mM, 1 mM, 10 mM and 100 mM) were examined also (Appendices I & J, respectively). Experiments were done three times in the case of NaCl and KCl and two times in the case of $MgCl_2$ and $MnCl_2$. Reactions were done in duplicate the first two times and in triplicate the third time in the case of NaCl and KCl. In the case of $MgCl_2$ and $MnCl_2$ the reactions were done in duplicate the first time and triplicate the second time. Activity corresponding to the 20 minute time point was plotted graphically.

Figure 12 shows maximum activity of tRNA nucleotidyltransferase at 100 mM NaCl. By 200 mM NaCl there is an apparent inhibitory effect on the activity of tRNA nucleotidyltransferase which is even more dramatic at 400 and 600 mM NaCl.

With respect to KCl concentrations tRNA nucleotidyltransferase had maximal activity between 0 and 200 mM. KCl concentrations of 400 and 600 mM were inhibitory to tRNA nucleotidyltransferase activity since these samples showed even less activity than that seen in the absence of KCl (Fig. 13).

Magnesium seems to be required for maximal activity of tRNA nucleotidyltransferase (Fig. 14). The enzyme showed a maximum activity at 10 mM Mg^{++}. Activity was less in the presence of 100 mM and 0.1 mM Mg^{++}, but still more, than when compared to the activity in the absence of magnesium ions.

Like magnesium, $MnCl_2$ stimulated the activity of tRNA nucleotidyltransferase (Fig.15). Although high activity was seen between 0.1 and

FIGURE 12

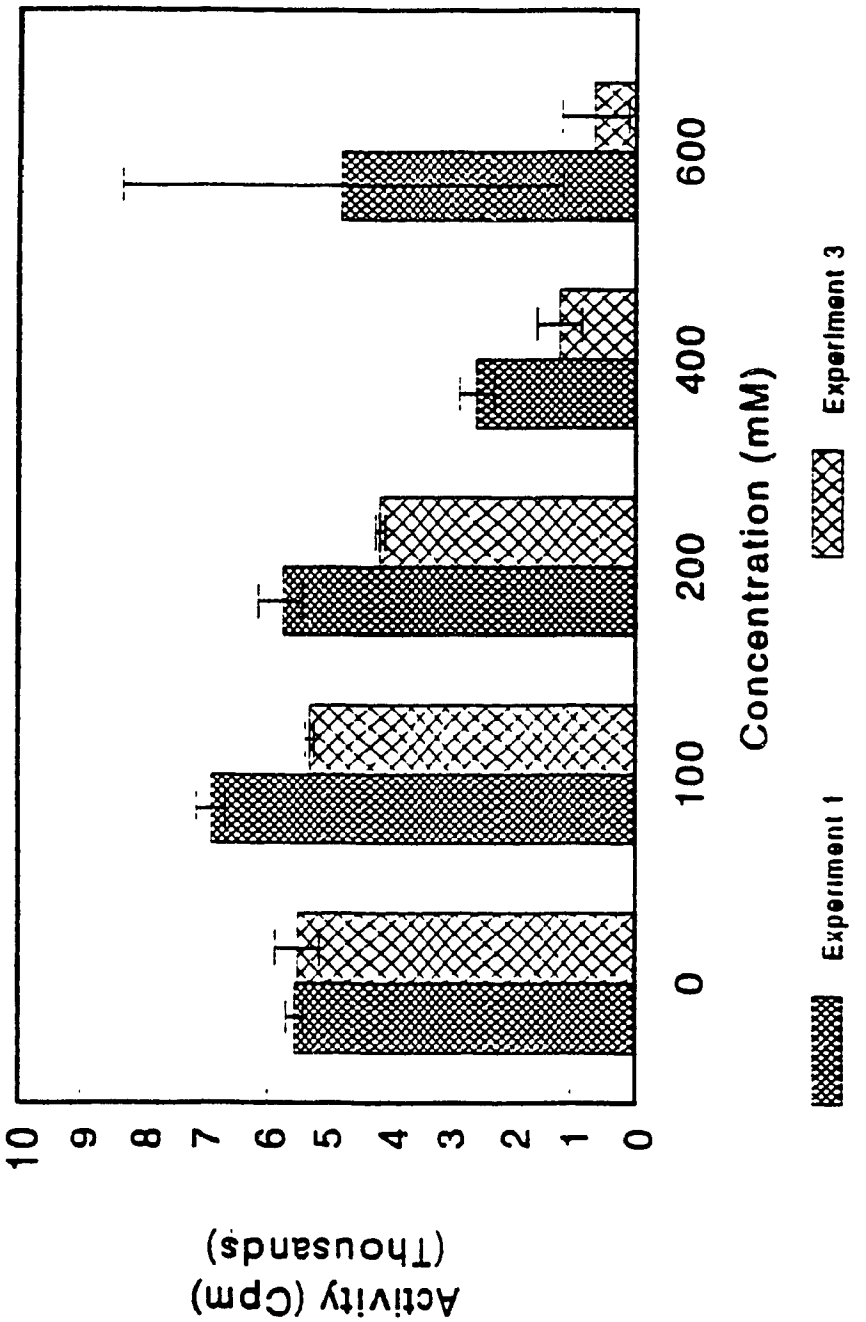

Effect of [NaCl] on the activity of tRNA nucleotidyltransferase
Activity (Cpm) (Thousands)
0
1
2
3
4
5
6
7
8
9
10
0
100
200
400
600
Concentration (mM)
Experiment 1
Experiment 3

FIGURE 13

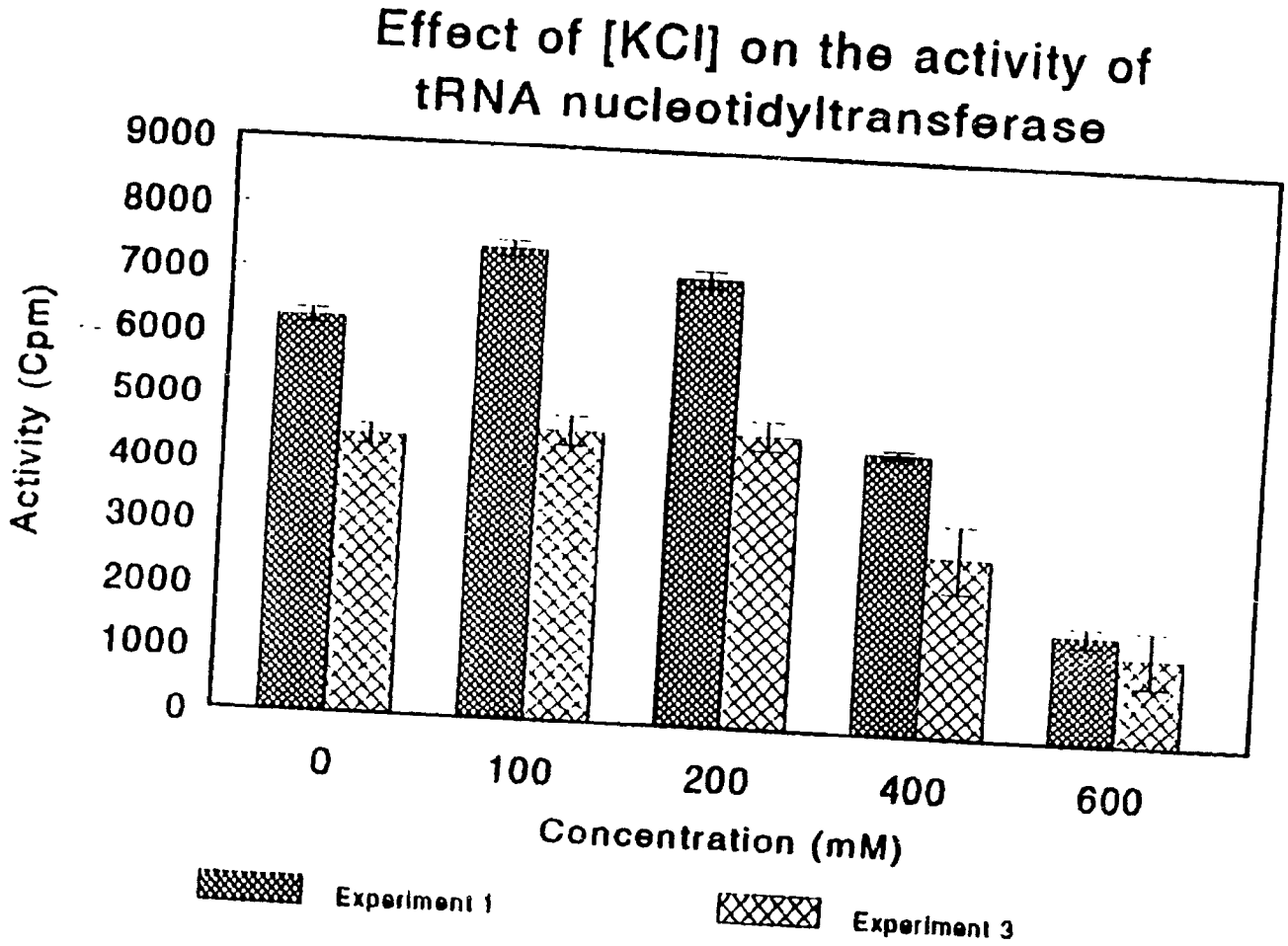

Effect of [KCl] on the activity of tRNA nucleotidyltransferase
Activity (Cpm)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
100
200
400
600
Concentration (mM)
Experiment 1
Experiment 3

FIGURE 14

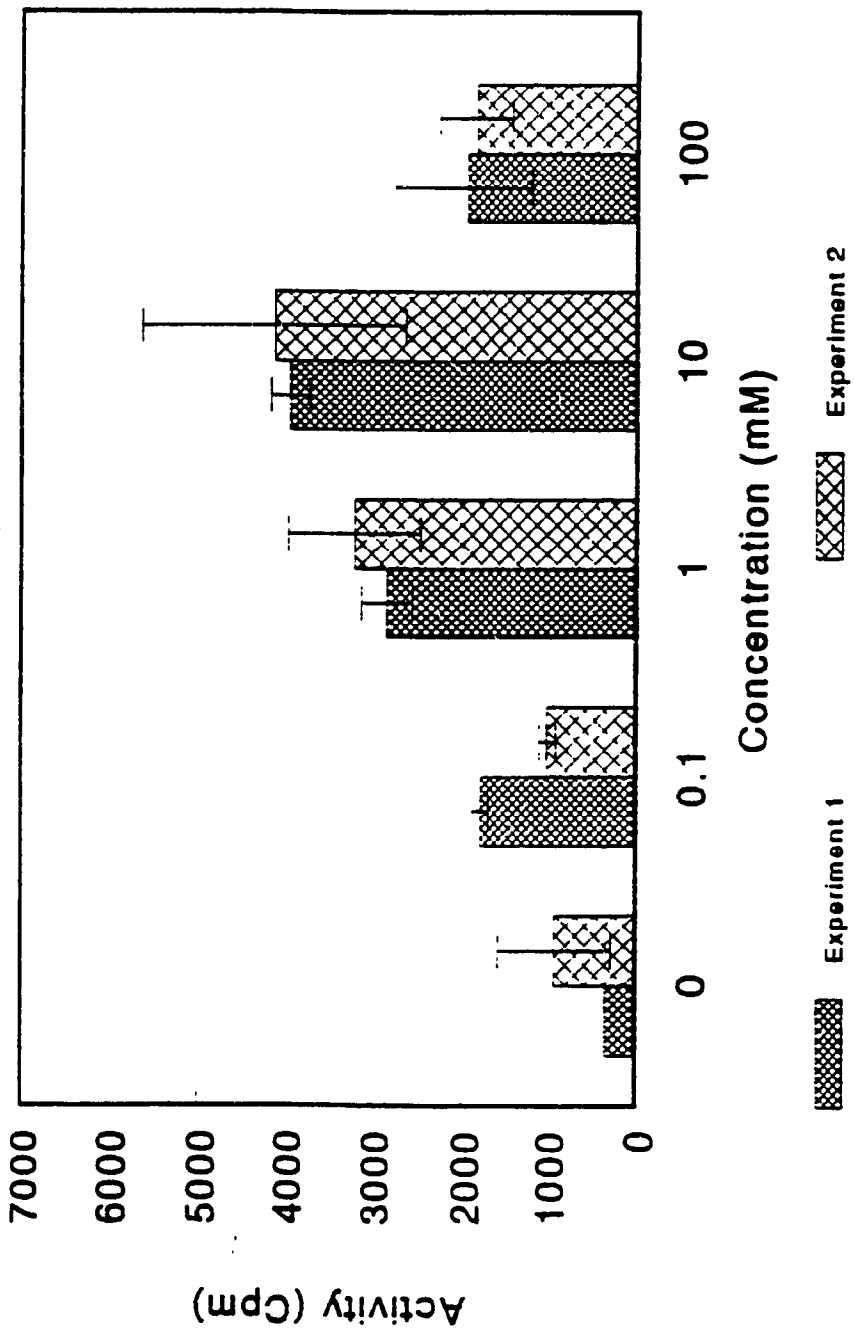
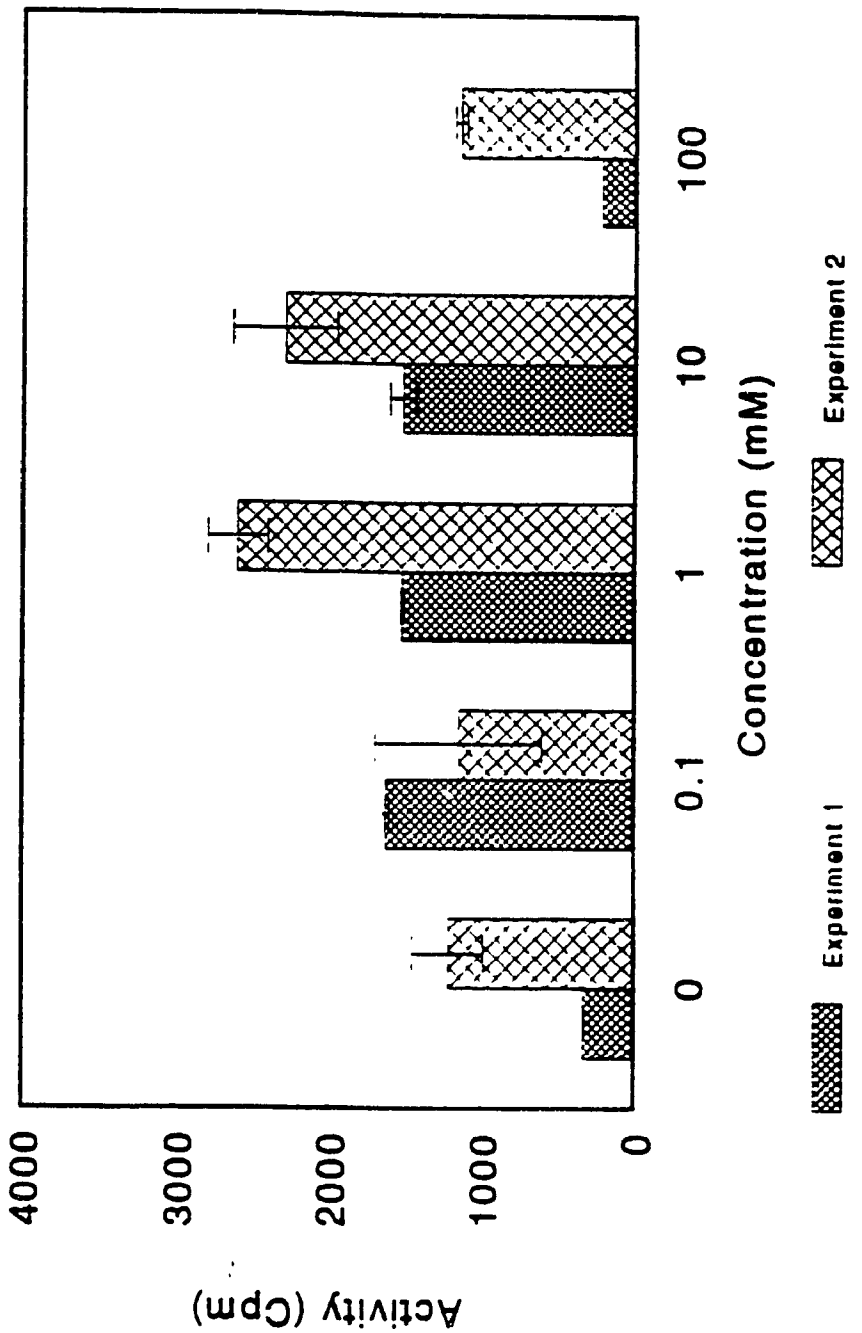

Effect of [MgCl2] on the activity of tRNA nucleotidyltransferase
Activity (Cpm)
7000
6000
5000
4000
3000
2000
1000
0
0
0.1
1
10
100
Concentration (mM)
Experiment 1
Experiment 2

FIGURE 15


Effect of [MnCl2] on the activity of tRNA nucleotidyltransferase
Activity (Cpm)
4000
3000
2000
1000
0
0
0.1
1
10
100
Concentration (mM)
Experiment 1
Experiment 2

10 mM $MnCl_2$, 1 mM $MnCl_2$ seems to be the optimum. Activity decreased below 0.1 mM and above 10 mM $MnCl_2$. In all experiments to determine the effect of $MnCl_2$, $MgCl_2$ was not added to the tRNA nucleotidyltransferase activity reaction mix.

G) Effect of EDTA

The trends suggested by these data (Fig. 16 and Appendix K) indicate that EDTA had an inhibitory effect on tRNA nucleotidyltransferase even at levels as low as 0.5 mM and 1 mM. At 10 mM EDTA, the enzyme activity was dramatically reduced. These reactions were carried out with 10 mM $MgCl_2$ in the reaction mix.

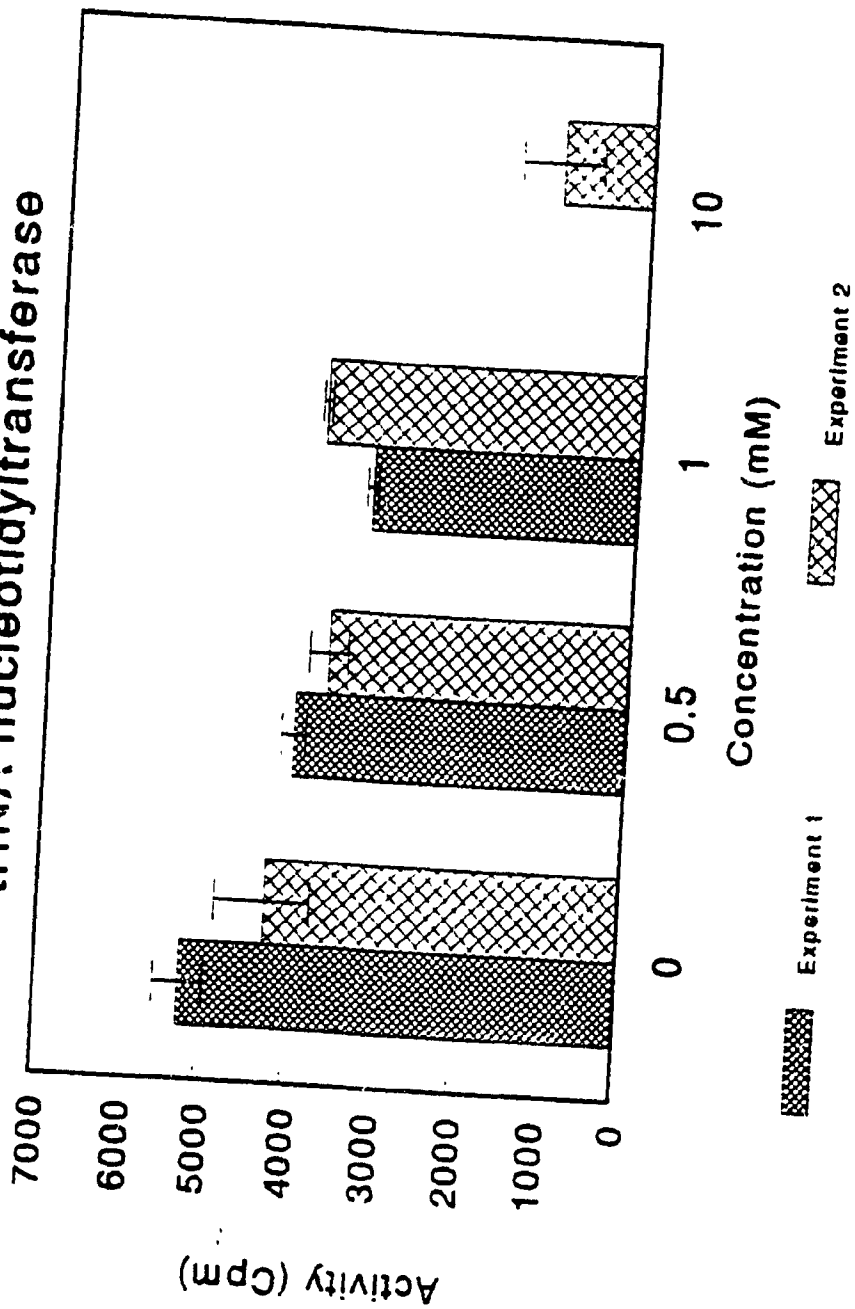
3) PEPTIDE SEQUENCE

The single band seen in lane 7 (Fig. 5) was transferred to PVDF membrane and sent to Harvard Microchem Facility, Harvard University. A tryptic digestion was performed on this and the size of the fragments checked by mass spectroscopy. Two independent peptides that arose from the tryptic digestion of pure tRNA nucleotidyltransferase were subjected to microsequencing and had the following amino acid sequence determined:

1) F G T P E E D A Y R R

2) D L T I N S L F Y N I N T D S V E D F T K R

FIGURE 16


Effect of [EDTA] on the activity of tRNA nucleotidyltransferase
Activity (Cpm)
7000
6000
5000
4000
3000
2000
1000
0
0
0.5
1
10
Concentration (mM)
Experiment 1
Experiment 2

4) CLONING

A) PCR

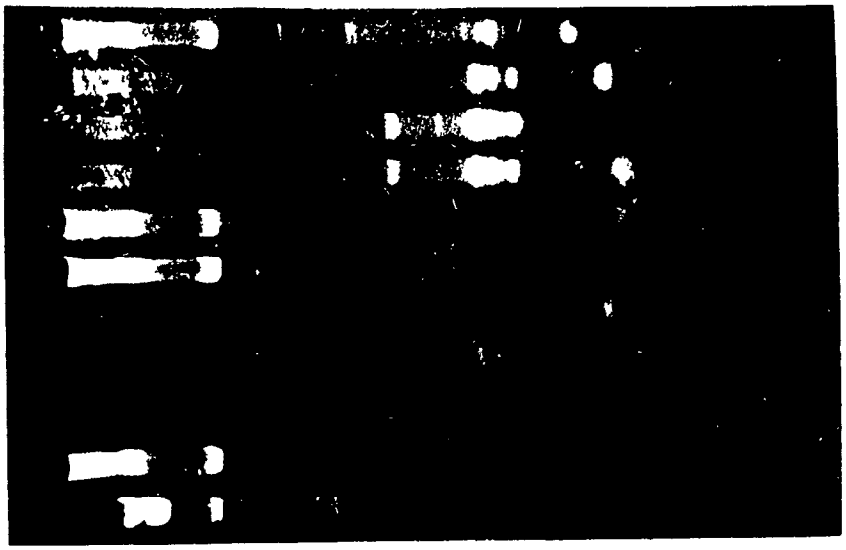
Polymerase chain reaction was performed on a lupin cDNA library and yeast genomic DNA (positive control) using degenerate oligonucleotides, CCA1 and CCA2 (see section II. 3. C) derived from the amino acid sequence of purified lupin tRNA nucleotidyltransferase that showed a high level of identity to the yeast homolog . The PCR products were run on a 1.5% agarose gel (Fig.17). Apart from the most prominent fragment of 80 base pairs in size in both the yeast and lupin reactions there were other bands that were seen in the same lane in proximity to the major fragment. Further characterisation of this prominent fragment (arrow in fig. 17) was carried out based on the known size of the yeast fragment, the predicted size of our insert and the fact that this prominent fragment was absent from the control lanes.

When this fragment was gel purified, cloned and sequenced, the following sequence was obtained:

TTT GGG ACG CCG GAA GAG GAT GCG TAT AGG AGG GAT TTG ACT ATT AAC AGC TTA TTT TAC AAC ATC AAC ACA GAC

The DNA sequence that we obtained was translated and compared to the sequence we had obtained from the tryptic fragments of the lupin tRNA nucleotidyltransferase. The sequence was identical over this region and showed good similarity also with the yeast tRNA nucleotidyltransferase, indicating that

FIGURE 17 PCR products from lupin cDNA library.



Photograph of an ethidium bromide stained 1.5% agarose gel showing the products of PCR obtained from a lupin cDNA library. The arrow marks the PCR product that was isolated, sequenced and used as a probe for hybridisation.

Lane 1: lambda DNA restricted with *Hin*dIII and *Eco*RI

Lane 4: cDNA library, primer CCA 2 alone, 30 cycles

Lane 5: cDNA library, primers CCA 1 and 2, 30 cycles

Lane 6: Bluescript, primers CCA 1 and 2, 30 cycles

Lane 9: cDNA library, primer CCA 2 alone, 40 cycles

Lane 10: cDNA library, primers CCA 1 and 2, 40 cycles

Lane 11: Bluescript, primers CCA 1 and 2, 40 cycles

Lanes 2,3,7and 8 are not relevant to the discussion.

we had indeed amplified a partial DNA fragment of cDNA encoding tRNA nucleotidyltransferase in lupin. The predicted translation product of the DNA sequence is as follows:

```
TTT GGG ACG CCG GAA GAG GAT GCG TAT AGG AGG GAT TTG ACT ATT
 F   G   T   P   E   E   D   A   Y   R   R   D   L   T   I
AAC AGC TTA TTT TAC AAC ATC AAC ACA GAC
 N   S   L   F   Y   N   I   N   T   D
```

B) Plaque hybridisation

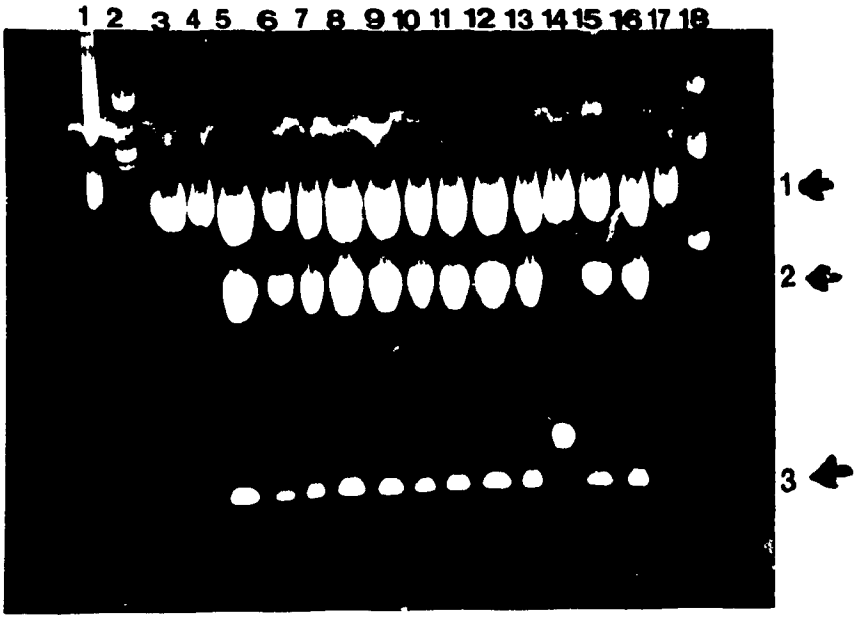
Screening of a total of 1 million cDNA clones (at a density of 50,000 plaques/plate on 137 mm plates) using the PCR amplified product as probe, gave 22 putative positive clones. Before proceeding with the characterisation of the 22 positives, 2 rounds of purification were carried out to avoid contaminating phage particles. At the end of these secondary screenings the number of positives was reduced to 16.

C) Restriction analysis

Restriction analysis performed on the 16 independent positives with restriction enzymes *Eco*RI and *Xho*I revealed a fragment of 500 basepairs in length, common to all of the 16 positive clones that were sequenced (Fig.18 and data not shown). This is likely to be in the 3' region of these clones since the *Eco*RI site in the forced cloning procedure used in constructing the library is at the 3' end and because the 5' region of the cDNA clones can be variable. This

Figure 18

Restriction analysis of positive cDNA clones



Photograph of an ethidium bromide stained 1.5% agarose gel showing the restriction products of some of the independent positive lupin tRNA nucleotidyltransferase cDNA clones. Arrow 1 marks the linearised plasmid, arrow 2 marks the larger fragment of the insert (around 1.5 kb) and arrow 3 marks the smaller fragment of the insert (around 500 bp).

Lane 1 - uncut plasmid (one of the positive clones)

Lane 2 - lambda DNA restricted with *Hin*dIII and *Eco*RI

Lanes 3 to 16 - positive clones digested with *Eco*RI and *Xho*I

Lane 17 - Bluescript digested with *Eco*RI and *Xho*I

Lane 18 - Bluescript uncut

common 500 bp fragment to some extent confirmed that they were all clones of the same gene. The second fragment that arose out of the restriction was in the range of 1450 - 1500 basepairs in all the clones (Fig.18).

5) cDNA SEQUENCE OF tRNA NUCLEOTIDYLTRANSFERASE

As a next step in confirmation, the 5' ends of the 16 clones were sequenced. Based on the length of their 5' sequences these cDNA's could be grouped into 1 of 6 families (Fig. 19). These families differed in length from a few bases to up to 80 bases at their 5' ends. Although these clones all showed similar sequence alignment, the two families of the longest cDNA's identified also showed sequence differences upstream of the first in frame start codon. These differences could be explained by a 1 base insertion, a 1 base deletion and an A to G or G to A transition. Sequence analysis of the 3' sequence of 5 of these clones revealed the same 3' sequence upstream of the poly A tail of each (Fig. 20).

The longest of these clones was chosen for complete sequence analysis. Deletions performed on both strands of this clone gave cDNA inserts carrying deletions in steps of 250-300 basepairs in length. A total of 7 deletion clones in the forward direction and 8 deletions in the reverse direction (Fig. 21) spanning the entire length of the cDNA clone were sequenced to determine the sequence of the full length cDNA encoding tRNA nucleotidyltransferase. Figure 22 shows the full length cDNA sequence of the longest of these clones (1.934 Kbp in length). The open reading frame starting from the first in frame methionine

FIGURE 19

Sequence of the 5' ends of the cDNAs showing positive hybridisation with PCR product

+1

1)TAAAGATTGAGTTGTTTAAGAGAAAGAAAC-AGAATCCCATGAGACTAAGTTTCAAAACTGTTAC (2 clones)

2) AAG-AACTAGAATCCCATGAGACTAAGTTTCAAAACTGTTAC (6 clones)

3) CCCATGAGACTAAGTTTCAAAACTGTTAC (5 clones)

4) ATGAGACTAAGTTTCAAAACTGTTAC (1 clone)

5) AGACTAAGTTTCAAAACTGTTAC (1 clone)

The in frame first ATG is in bold

The sixth family consisting of one clone starts at

position + 68 in the cDNA sequence.

FIGURE 20

Sequence of the 3' ends of 5 clones showing positive hybridisation with PCR product

A) 5' TTTTTATTTATAAAAACTATATTAGAAATCCA 3'

B) TTTTTATTTATAAAAACTATATTAGAAATCCA

C) TTTTTATTTATAAAAACTATATTAGAAATCCA

D) TTTTTATTTATAAAAACTATATTAGAAATCCA

E) TTTTTATTTATAAAAACTATATTAGAAATCCA

The last A at the 3' end represents the first A of the poly A tail.

The above 3' end sequences represent families 1, 2 and 4 from Fig.19.

Sequences A, B and C are from family 1 while sequence D is from family 2 and sequence E is from family 4.

FIGURE 21

NESTED DELETIONS OF CLONE 9_{C3}

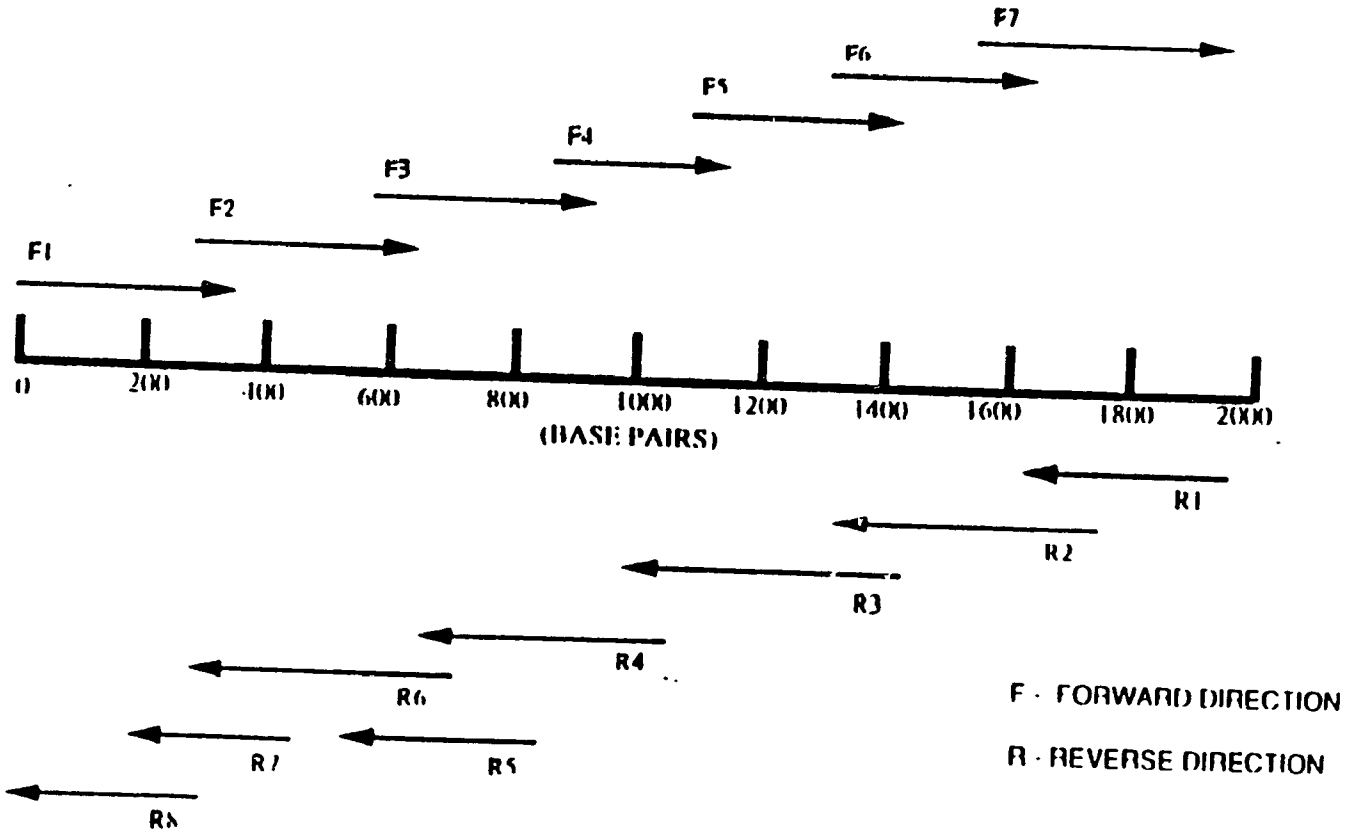


FIGURE 22:
Full length cDNA sequence of tRNA nucleotidyltransferase

TAAAGATTGAGTTGTTTAAGAGAAAGAAACAGAATCCCATGAGACTAAGTTTCAAAACTG
TTACAAACGTTGTTGTTGTGCTTCCTAGAGGCAGAACTAGAAGCATCATTAACTTCACCC
TCTTCCCAACCATTACTTCAAATCTCGTTCTTCATCCTCTTCTCCGCACTCCCAAAACGC
CCTCGTTTCACTCTTCTCTCTCCTCACCAATGTCTTCACACAAGGTTCGAGACAATATTC
AACTCTCCGACGTAGAGAAGAGAATCTTCGATAGGCTTCTCGCTACTCTCCGCTTTTTCA
ACCTCCAAACTCACCTTCGTGTTGCGGGTGGCTGGGTTCGCGACAAGCTTCTTGGAAAAG
AATGCTATGACATTGATATTGCACTTGACAAGATGATGGGAACTGAGTTTGTGGATAAGG
TTAGGGAATATTTGTTATCCATTGGTGAAGAAGCACAAGGTGTTTGTGTTATTGAAAGCA
ACCCTGACCAGTCCAAACATTTGGAAACAGCAAGGATGCGATTATTTGATATGTGGATTG
ATTTTGTTAACTTAAGGAGTGAAGAGTACACCGATAATAGCCGCATCCCCTCTATGCAAA
GATTTGGCACACCTGAAGAGGATGCGTATAGGAGGGATTTGACTATTAACAGCTTATTCT
ACAATATCAACACCGATTCAGTTGAAGATTTTACTAAGAGAGGGATCTCAGACCTTAAAT
CTGGAAAGATAGTAACTCCCTTACCTCCAAAGGCCACATTTCTTGATGATCCCTTACGAG
TTGTTCGAGCCATTCGATTTGGTGCTCGATTTGAATTTACTCTAGATGAAGATCTGAAAC
AAGCTGCTGCATGTGATGAAGTAAAGGATGCATTAGCTGCTAAAATTAGCCGAGAGCGCA
TTGGAACAGAGATTGATCTTATGATATCTGGAAATCAACCTGTCAAAGCAATGACTTATA
TTTGTGACCTCACAATATTTTGGATTGTATTCAGTCTTCCTCCTACGTTTGAACCTGCCA
TCTCAGATGGATGTGAAAGGCTTTGCATTTCTCAATTGGATATCTCATGGAACCTTATCC
ATTTACTAGGAAAGACCACCTTTACAGATGAACAAAGAAGGTTAACACTTTATGCTGCTA
TGTTTCTCCCACTGAGAAATACCATTTACAGAGAAAAGAAGGCTAAAAAGGTTCCCGTTG
TCAATTATATTTTCCGCGAATCTCTCAAGCGAAAAGCTAAGGATCCAGAAACGGTGCTTG
ATTTACACCGAGCATCAAATAAATTCTTGTCGTTAATTCCATGTCTTGTATCTAATGAGG
ATGTCCAAATTGTTGGTCACGATTGGATGACAGAATTGATTGATGTCCCTGTCTCTTCTA
GAGTCCGGGTTCTAACAGGGTTTCTTTTGAGAGAGCTTAGAGATTTTTGGCGAGTTGCAT
TATTGATATCCATATTATTACATCCCATTGACGTTAACGATACCGAAGATGAGTCATCTC
AGTTGAGCAAACGAAGGGATCTGTTTAATACCGTGGAGAATTCTGTAATCAAACTAGGCC
TTGAGAAAGTATGGGACGTAAAGCAATTGATAAATGGGAAAGATGTGATGAGTGTCTTGC
AGCTTAAAGGAGGACCTATGGTTAAGGAATGGCTAGATAAAGCAATGGCTTGCAACTTG
CCCATCCCTCAGGAACTGCAGAGGAATGTCTTGATTGGTTGAGAGAAGCCAATTCTAAGC
GTGTAAAGTTGGAGTGAGTGAGTGAGGTTGAATACTCATCAATTTTCTATTCCTCACTTG
CAACTGTATAAAGCCTCAAGTAGGAACTAGTTCATTCTCAGATTTAGATATCAGGATCAA
TTTTTATGTAGTGATGTTCTTGTACTCTTAAATACCTCACATTTTTTATTTATAAAACT
ATATTAGAAATCC

(underlined at position 39) to the first in frame stop codon (double underlined at position 1719) was exactly 1.680 Kbp in length and could code for a protein of 560 amino acids. A potential polyadenylation signal (Bold in figure 22) is present 22 bases upstream of the poly A tail (Joshi, 1987).

IV. DISCUSSION

This study was started with the aim of isolating a full length cDNA clone encoding tRNA nucleotidyltransferase from *Lupinus albus*. The approach that was chosen to isolate the cDNA encoding tRNA nucleotidyltransferase was to first purify the tRNA nucleotidyltransferase from *Lupinus albus* and then to use partial peptide sequence obtained from this protein to construct oligonucleotides for use as PCR primers. This method was used to isolate the cDNA encoding lupin tRNA nucleotidyltransferase because other methods like heterologous hybridisation using the yeast gene as probe and complementation of a yeast temperature-sensitive mutant were unsuccessful.

The results of this study are discussed under three broad headings, namely, 1) purification of tRNA nucleotidyltransferase, 2) characterisation of tRNA nucleotidyltransferase and 3) isolation of tRNA nucleotidyltransferase cDNA.

1. PURIFICATION OF tRNA NUCLEOTIDYLTRANSFERASE

A) Purification

The 30 - 55% ammonium sulphate fractionation resulted in a 2 fold purification of the tRNA nucleotidyltransferase from crude extracts (Table 1). Subsequent purification by DEAE column chromatography achieved a 7.5 - 9 fold purification (Table 1) similar to that achieved by Cudny *et al.* (1978-A) at this

step. Active fractions at the end of DEAE chromatography were devoid of background activity in the absence of added tRNA substrate likely due to a lack of endogenous tRNA. This is also in agreement with the findings of Cudny *et al.* (1978-A) because tRNAs do not bind to the DEAE resin which is an anion exchanger. This also suggests that the protein purified in this study is negatively charged and binds weakly to the DEAE resin since it eluted in low salt, *i.e.*, 60 mM KCl.

Hydroxylapatite chromatography following the DEAE chromatography resulted in a 222 fold purification over the crude extract (Table 1). This is a large increase over what was achieved by Cudny *et al.* (1978-A) at the same point in their purification scheme (63.7 fold). Though the amount of starting material in this study and that of Cudny *et al.* (1978-A) were the same, the difference in the fold purification could be due to the fact that in this study protein was eluted with a broader phosphate buffer gradient from 10 mM to 250 mM (500 ml) than in the case of Cudny *et al.* (1978-A) where a narrower phosphate gradient from 20 mM to 150 mM (1 l) was used. Also in the case of Cudny *et al.* (1978-A) active fractions from the DEAE column were pooled, brought to 60 % ammonium sulphate concentration, precipitated by centrifugation, resuspended in 10 mM phosphate buffer and dialyzed against the same buffer before being loaded on the HA column. It is possible that the above procedure resulted in loss of activity in contrast to our procedure where the pooled fractions from DEAE chromatography were dialysed against 10 mM phosphate buffer and loaded

directly onto the HA column.

The tRNA-Sepharose affinity chromatography used with the DEAE and HA columns resulted in a 5575 fold purification over the 30 - 55% fraction. Comparing the specific activity and the fold purification achieved by us in this study to those discussed above, it seems that the purification in this study resulted in a better yield of tRNA nucleotidyltransferase with a better specific activity than those purified by earlier workers from lupin, wheat, yeast and *E. coli*. This also suggests that the affinity resin gave the greatest fold purification of tRNA nucleotidyltransferase. From figure 3 it is also clear that tRNA nucleotidyltransferase has a high affinity for the ligand (total wheat tRNA) and requires 200 mM NaCl to be eluted.

B) Molecular weight of lupin tRNA nucleotidyltransferase

The apparent molecular weight of the purified tRNA nucleotidyltransferase from *Lupinus albus* based on SDS polyacrylamide gel electrophoresis is approximately 64 000 Daltons. The yeast tRNA nucleotidyltransferase had an apparent molecular weight of 59 000 Daltons (Chen *et al.*, 1990). In contrast, the rabbit liver tRNA nucleotidyltransferase had an apparent molecular weight of 47 000 Daltons on SDS PAGE (Deutscher, 1972-A) while the apparent molecular weight of the *E. coli* tRNA nucleotidyltransferase on SDS PAGE was 51 000 Daltons (Schofield and Williams, 1977). This value for the *E. coli* enzyme was in good agreement with the molecular weight of the native form (53 000 Daltons)

based on gel filtration chromatography (Schofield and Williams, 1977). The molecular weight of the wheat tRNA nucleotidyltransferase was not determined (Dullin *et al.*, 1975). Cudny *et al.* (1978-B) reported tRNA nucleotidyltransferase activity from *L. luteus* correlating with a single protein band on an SDS polyacrylamide gel, however, they did not include size markers on their gel. Hence, it is impossible to accurately determine the size of the single band they correlated with activity.

Cudny *et al.* (1978-A) report the native molecular weight for *Lupinus luteus* tRNA nucleotidyltransferase at around 40 000 + or - 5000 Daltons. The above data could not be compared to the present study since the molecular weight of tRNA nucleotidyltransferase purified in this study was not estimated in its native form. Taken together with the sizes predicted for tRNA nucleotidyltransferases from other sources our results are in good agreement. Our protein appears to be more similar in apparent molecular weight to the yeast enzyme than to the *E. coli* enzyme. This similarity in molecular weight with the yeast enzyme is also reflected in the amino acid identity between these proteins in that the predicted lupin amino acid sequences are more similar to those of yeast than to *E. coli*.

C) Protein sequence of tRNA nucleotidyltransferase

Partial peptide sequences, FGTPEEDAYRR and DLTINSLFYNINTDSVEDFTKR, derived from sequencing of two independent tryptic fragments of lupin tRNA nucleotidyltransferase purified in this study were

used to search the GenBank database with the BLASTX (Gish and States, 1993, and Altschul *et al.*, 1990) search program which compares the query sequence to existing similar protein sequences submitted to the GenBank.

The only match the above sequences picked up was the yeast tRNA nucleotidyltransferase. When the complete lupin cDNA sequence was sent to search the BLASTX database for similar proteins it picked up the *E. coli* sequence in addition to the yeast sequence.

The following is the alignment of the two lupin tRNA nucleotidyltransferase partial peptide sequences with the yeast tRNA nucleotidyltransferase (Aebi *et al.*, 1990) showing the degree of identity between the two protein sequences:

```
1)   Lupin    1  FGTPEEDAYRR  11
                 ||||||| ||
     Yeast  162  FGTPEEDALRR  172

2)   Lupin    1  DLTINSLFYNINTDSVEDFTKR  22
                 | | | |||||    |||||||
     Yeast  173  DATLNALFYNIHKGEVEDFTKR  194
```

Both the sequences derived from lupin tRNA nucleotidyltransferase showed a high degree of identity with the yeast tRNA nucleotidyltransferase

(Aebi *et al.*, 1990).

The two yeast tRNA nucleotidyltransferase amino acid sequences (162-172 and 173-194) similar to our lupin peptides were adjacent in the yeast protein and provided a stretch of 33 contiguous amino acids. Based on this observation, we hypothesised that a similar organisation would exist in the lupin protein. This 33 amino acid stretch in the yeast sequence spanned amino acids 162-194 predicted from the yeast sequence (Aebi *et al.*, 1990). As the yeast open reading frame codes for a protein of 547 amino acids this represents a portion of the protein at about one third the distance from the amino terminus.

2. CHARACTERISATION OF LUPIN tRNA NUCLEOTIDYLTRANSFERASE

In this study total wheat tRNA was used as a substrate for tRNA nucleotidyltransferase in all experiments. These tRNAs were not specifically treated to remove the 3' terminal CCA that may have existed due to the endogenous enzyme so one would expect a mixed population of tRNAs, *i.e.*, the 3' end of tRNAs may contain either N-, N-C, N-C-C or a complete N-C-C-A. Because this is the case precise kinetic parameters regarding the incorporation of labeled ATP could not be determined since we did not know exactly how many tRNAs in our population required ATP addition. However, since the same general population of wheat tRNAs was used in each experiment the general conclusions drawn are valid.

A) Time course

The effect of incubation time on the activity of tRNA nucleotidyltransferase in this study revealed that incorporation of ATP was linear with time until 10 minutes in every experiment we conducted (Fig. 6). The activity plateaued after 15-30 minutes incubation, indicating that the enzyme had used up most of the substrate. This is in line with the time course data of Dullin *et al.* (1975) for tRNA nucleotidyltransferase from wheat embryos. Compared to the 20 μg of tRNA used as substrate in our reactions to measure the activity of tRNA nucleotidyltransferase Dullin *et al.* (1975) used 5 μg of yeast tRNA with partially degraded 3' terminus in their reaction mix. They also carried out their experiments at 30^{0}C as opposed to 21^{0}C used in this study. In comparison the increase in activity of the *E. coli* tRNA nucleotidyltransferase (Williams and Schofield, 1977) seems to be linear up to 30 minutes. Williams and Schofield (1977) used *E. coli* tRNA with 3' ends degraded by treatment with snake venom phosphodiesterase. In their experiments the ATP incorporation rate seemed to deviate from the linear plot gradually after 30 minutes which is in contrast to 15-20 minutes in this study. This may be because the *E. coli* tRNA nucleotidyltransferase has a lower reaction velocity or there is more substrate still left for the enzyme to act on or more enzyme was used in the enzyme assay in this study. Williams and Schofield (1977) used 100 μg of tRNA (yeast) as opposed to 20 μg in the assay reaction mix used in this study. We chose to use the 20 minute time point in all further experiments since this was near the linear

portion of the graph.

B) Buffer concentration

The optimum glycine buffer concentration (Fig. 7) arrived at in this study (100 mM) does not agree with that reported by Cudny *et al.* (1978-A) for the *Lupinus luteus* enzyme. No marked influence on activity at concentrations greater than 100 mM was noticed in this study, although Cudny *et al.* (1978-A) reported that concentrations greater than 100 mM markedly influenced enzyme activity in that they saw optimum activity at 400 mM glycine. The above difference could be attributed to the difference in the assay conditions for tRNA nucleotidyltransferase. Another possibility is that *Lupinus luteus* enzyme has a buffer optimum of 400 mM glycine. From our results in this study we used glycine at a concentration of 100 mM for all the characterisations.

C) pH optimum

We report a pH optimum of 9.0 - 9.5 for tRNA nucleotidyltransferase. Williams and Schofield (1977) reported a similar pH optimum of 9.0 for *E. coli* tRNA nucleotidyltransferase. Rabbit liver tRNA nucleotidyltransferase had a pH optimum range of 9.3 - 10 (Masiakowski and Deutscher, 1980), which is similar to what we observed in this study. The lupin and the yeast tRNA nucleotidyltransferases had pH optima of 9.5 (Cudny *et al.*, 1978-A, Chen *et al.*, 1990). All of the tRNA nucleotidyltransferase enzymes described above have a pH optimum between pH 9 and pH 9.5 in good agreement with our data. In

contrast to the enzymes described above, Dullin *et al.* (1975) indicated that tRNA nucleotidyltransferase from wheat embryos had a maximum activity at pH 7.6 with half-maximal activity at pH 8.6. It is possible that wheat tRNA nucleotidyltransferase was at its maximum activity at pH 7.6 because the buffer used by Dullin *et al.* (1975) was Tris-HCl as opposed to glycine used in this study and by earlier workers. However, we also observed a pH optimum of 8 - 8.5 in this study with Tris-HCl buffer (data not shown). Another possible explanation for the results observed by Dullin *et al.* (1975) is that they isolated a different tRNA nucleotidyltransferase isozyme. Masiakowski and Deutscher (1980) showed two tRNA nucleotidyltransferases active in rabbit liver, presumably one working in the nucleus and the other in the mitochondrion both with a pH optimum between 9.3 - 10. One possible explanation for Dullin's finding of two pH optima for wheat tRNA nucleotidyltransferase could be that they represent seperate nucleocytoplasmic and mitochondrial forms of the enzyme.

D) Effect of ATP and CTP

In the presence of 0.015 μM [α^{32}P] ATP, maximum incorporation of radioactive ATP was shown at 0 mM non-radioactive ATP. As the concentration of non-radioactive ATP increased the apparent incorporation of radioactive ATP decreased (Fig. 9). This is due to competition *in vitro* between the [α^{32}P] ATP and the non-radioactive ATP. This could not be compared to the standard assay for tRNA nucleotidyltransferase of Cudny *et al.* (1978-A), since they used a total

of 0.2 mM radioactive ATP.

Cold CTP at differing concentrations had a similar effect as ATP did on the incorporation of [α^{32}P] ATP. Maximum incorporation seen with concentrations as low as 0.01 mM and 0.1 mM decreased as the concentration of CTP increased to 1 mM. Incorporation was very low or completely abolished at 10 mM CTP (Fig. 10). This decrease in incorporation of ATP with increasing CTP levels could be due to CTP competing for addition at the third position, which would result in CCC instead of CCA at the 3' end of tRNA. Miller and Philipps (1971) showed that high concentrations of CTP competetively inhibit the incorporation of ATP into tRNA-C-C. These data could not be compared to that of Cudny *et al.* (1978-A) since they used a total of 0.2 mM radioactive CTP in their assay for tRNA nucleotidyltransferase activity as opposed to 0.0154 μM [α^{32}P]ATP with which the assay conditions were standardised in this study.

E) Effect of temperature

The maximum enzyme activity seen at 45^{0}C (Fig. 11) is in line with that reported by Cudny *et al.* (1978-A) for the *Lupinus luteus* enzyme. At 65^{0}C, the activity decreased rapidly within 20 minutes. This also agrees with the findings of Cudny *et al.* (1978-A) for the *Lupinus luteus* enzyme. This shows that tRNA nucleotidyltransferases from different species of the same plant share a similar temperature optimum.

F) Effect of metal ions

The effect of KCl on the activity of tRNA nucleotidyltransferase in this study showed that concentrations above 100 mM are inhibitory to the activity of the *L. albus* tRNA nucleotidyltransferase (Fig. 13). This agrees with that reported by Cudny *et al.* (1978-B) for the *Lupinus luteus* enzyme. In the case of wheat mitochondrial tRNA nucleotidyltransferase Hanic-Joyce and Gray (1990) also reported an inhibitory effect on the activity of the protein in the presence of 200 mM KCl although 50 -150 mM KCl did not stimulate the activity of that protein. NaCl showed an effect similar to KCl in this study (Fig. 12).

The requirement for $MgCl_2$ or $MnCl_2$ for maximal activity of tRNA nucleotidyltransferase shown in this study is similar to that reported by Cudny *et al.* (1978-B) and Dullin *et al.* (1975) for the *Lupinus luteus* and wheat enzymes, respectively. The optimum concentrations of Mg^{++} and Mn^{++} required were 10 mM and 1 mM respectively (Figs. 14 & 15). Concentrations above 10 mM $MgCl_2$ were inhibitory to the activity of the enzyme. From this experiment it is possible to infer that either one of these divalent cations may be used in the reaction to stimulate the activity of tRNA nucleotidyltransferase. Since the divalent cations were tried individually in the reaction to measure the activity of tRNA nucleotidyltransferase it is not possible to say what effect they would have if used together in the reaction mix.

G) Effect of EDTA

Inhibition of tRNA nucleotidyltransferase activity by EDTA at low concentrations (0.5 mM) is in line with that reported by Cudny *et al.* (1978-B) for the *Lupinus luteus* enzyme. It is possible that this effect is seen because EDTA chelates the divalent cations present in the reaction mix which are essential for the activity of tRNA nucleotidyltransferase.

3. CLONING OF tRNA NUCLEOTIDYLTRANSFERASE

A) Polymerase chain reaction

The amino acid sequence predicted from the 75 bp DNA fragment amplified by PCR (section III. 4. A) was identical to the protein sequence determined from Edman degradation of the tRNA nucleotidyltransferase peptides (section III. 3).

B) Full length cDNA clone

This PCR product was subsequently used to screen a lupin cDNA library and its sequence shown to be identical to nucleotides 564-639 (Fig.22) of the cDNA that was completely sequenced from this library (with the exception of the positions where any one of the four bases or inosines were used in the PCR primers). When the sequence of this cDNA was analysed with the BLASTN program which selects high levels of nucleotide sequence similarity no matches were reported. However, when analysed with BLASTX or TBLASTN which compares protein sequences, yeast tRNA nucleotidyltransferse as well as the *E.*

coli enzyme were scored as matches. These results suggested that although lupin and yeast shared sequence similarity at the amino acid level, they lacked similarity at the DNA level. This finding may help to explain why heterologous hybridisation using the yeast gene as probe was unsuccessful. Due to the difference in codon usage in plants and yeast, the lupin cDNA sequence was different from the yeast sequence. The predicted protein sequence from the longest open reading frame begining with an ATG of a tRNA nucleotidyltransferase cDNA had a total of 560 amino acids (Fig. 23). This size is similar to the yeast tRNA nucleotidyltransferase protein sequence which contains 546 amino acids (Aebi *et al.*, 1990). The longest clone that was completely sequenced did not have a stop codon upstream of the first in frame ATG which would give a definitive indication that we indeed have the full length clone, *i.e.*, we have a complete 5' end. A second clone whose 3' end sequence was identical (Fig. 20) had a 5' region (Fig. 19) that differed at 3 positions. One of these differences suggested either the creation of a stop codon upstream of the first in frame ATG in this clone or the loss of a stop codon from the first clone. Complete characterisation of this clone is in progress. Thus far more than 1400 bases of this clone have been sequenced and there do not seem to be any other differences (F Khoubehi and A Chang, personal communication). This suggests that the differences in the 5' regions of these cDNA may be cloning artifacts perhaps produced by errors in reverse transcription while making the cDNA library from mRNA. It would be of interest to determine which of these

FIGURE 23: Predicted amino acid sequence of tRNA nucleotidyltransferase

Met Arg Leu Ser Phe Lys Thr Val Thr Asn Val Val Val Val Leu 15

Pro Arg Gly Arg Thr Arg Ser Ile Ile Asn Phe Thr Leu Phe Pro 30

Thr Ile Thr Ser Asn Leu Val Leu His Pro Leu Leu Arg Thr Pro 45

Lys Thr Pro Ser Phe His Ser Ser Leu Ser Ser Pro Met Ser Ser 60

His Lys Val Arg Asp Asn Ile Gln Leu Ser Asp Val Glu Lys Arg 75

Ile Phe Asp Arg Leu Leu Ala Thr Leu Arg Phe Phe Asn Leu Gln 90

Thr His Leu Arg Val Ala Gly Gly Trp Val Arg Asp Lys Leu Leu 105

Gly Lys Glu Cys Tyr Asp Ile Asp Ile Ala Leu Asp Lys Met Met 120

Gly Thr Glu Phe Val Asp Lys Val Arg Glu Tyr Leu Leu Ser Ile 135

Gly Glu Glu Ala Gln Gly Val Cys Val Ile Glu Ser Asn Pro Asp 150

Gln Ser Lys His Leu Glu Thr Ala Arg Met Arg Leu Phe Asp Met 165

Trp Ile Asp Phe Val Asn Leu Arg Ser Glu Glu Tyr Thr Asp Asn 180

Ser Arg Ile Pro Ser Met Gln Arg Phe Gly Thr Pro Glu Glu Asp 195

Ala Tyr Arg Arg Asp Leu Thr Ile Asn Ser Leu Phe Tyr Asn Ile 210

Asn Thr Asp Ser Val Glu Asp Phe Thr Lys Arg Gly Ile Ser Asp 225

Leu Lys Ser Gly Lys Ile Val Thr Pro Leu Pro Pro Lys Ala Thr 240

Phe Leu Asp Asp Pro Leu Arg Val Val Arg Ala Ile Arg Phe Gly 255

Ala Arg Phe Glu Phe Thr Leu Asp Glu Asp Leu Lys Gln Ala Ala 270

Ala Cys Asp Glu Val Lys Asp Ala Leu Ala Ala Lys Ile Ser Arg 285

Glu Arg Ile Gly Thr Glu Ile Asp Leu Met Ile Ser Gly Asn Gln 300

Pro Val Lys Ala Met Thr Tyr Ile Cys Asp Leu Thr Ile Phe Trp 315

Ile Val Phe Ser Leu Pro Pro Thr Phe Glu Pro Ala Ile Ser Asp 330

Gly Cys Glu Arg Leu Cys Ile Ser Gln Leu Asp Ile Ser Trp Asn 345

Leu Ile His Leu Leu Gly Lys Thr Thr Phe Thr Asp Glu Gln Arg 360

Arg Leu Thr Leu Tyr Ala Ala Met Phe Leu Pro Leu Arg Asn Thr 375

Ile Tyr Arg Glu Lys Lys Ala Lys Lys Val Pro Val Val Asn Tyr 390

Ile Phe Arg Glu Ser Leu Lys Arg Lys Ala Lys Asp Pro Glu Thr 405

Val Leu Asp Leu His Arg Ala Ser Asn Lys Phe Leu Ser Leu Ile 420

Pro Cys Leu Val Ser Asn Glu Asp Val Gln Ile Val Gly His Asp 435

Trp Met Thr Glu Leu Ile Asp Val Pro Val Ser Ser Arg Val Arg 450

Val Leu Thr Gly Phe Leu Leu Arg Glu Leu Arg Asp Phe Trp Arg 465

Val Ala Leu Leu Ile Ser Ile Leu Leu His Pro Ile Asp Val Asn 480

Asp Thr Glu Asp Glu Ser Ser Gln Leu Ser Lys Arg Arg Asp Leu 495

Phe Asn Thr Val Glu Asn Ser Val Ile Lys Leu Gly Leu Glu Lys 510

Val Trp Asp Val Lys Gln Leu Ile Asn Gly Lys Asp Val Met Ser 525

Val Leu Gln Leu Lys Gly Gly Pro Met Val Lys Glu Trp Leu Asp 540

Lys Ala Met Asp Cys Asn Leu Pro Ile Pro Gln Glu Leu Gln Arg 555

Asn Val Leu Ile Gly 560

Two potential nuclear localisation signals (amino acids 381-384 and 398-401) are double underlined.

two sequences represents the real transcript from the lupin CCA gene. If the TAG stop codon is real then this would suggest that we have a full-length cDNA for the lupin CCA gene. If, however, the TAG had resulted from a cloning artifact then it is possible that the lupin cDNA may be longer and could potentially encode several more amino terminal amino acids. This is of particular interest to us since we are interested in determining whether or not this protein may contain an amino terminal mitochondrial or chloroplast targeting signal. To overcome this problem Northern hybridisation or primer extension analysis may help to determine the size of the mRNA produced from the CCA gene. If the transcript seems to be significantly longer than the cDNA in this study 5' RACE can be performed to determine the remaining nucleotides at the 5' region. RACE is rapid amplification of cDNA ends wherein the 5' region of a gene can be amplified from mRNA to get first strand cDNA followed by subsequent amplification of this first strand cDNA. The significance of having a full length cDNA clone is very important in the context of protein targeting because previous studies (Chen *et al.*, 1992) on the yeast tRNA nucleotidyltransferase have shown that the mitochondrial import signal is at the amino terminus of this protein. That both of these cDNAs encode a functional tRNA nucleotidyltransferase is evident from the fact that both of the clones complement a temperature-sensitive mutation in the yeast gene that encodes tRNA nucleotidyltransferase (personal communication, P. J. Hanic-Joyce). This shows that the protein produced by either of these two cDNAs can function in yeast. Because we were able to

complement the yeast mutation with a lupin cDNA this suggests that the failure to clone this gene by complementation of the yeast mutation using the *Arabidopsis* library was due to the quality of the *Arabidopsis* library itself. Sequence analysis of other cDNA clones from the *Arabidopsis* library (B. Martin, personal communication) suggest that there are few full-length clones in it. Many plant genes have a consensus around the translation start site in that they have an A at -3 position and a G at the +4 position (Heidecker and Messing, 1986). On the contrary no such consensus was seen the clones characterised in this study. However, it is not possible to say whether or not we have the full length clone based on this observation since this consensus is not found around the start codons of all plant genes (Heidecker and Messing, 1986).

The predicted molecular weight of the protein from the ORF of the cDNA was 64 164 Daltons which agrees with the molecular weight based on SDS polyacrylamide gel electrophoresis in this study (64 000 Daltons). It is also in line with the predicted molecular weight of yeast tRNA nucleotidyltransferase reported by Aebi *et al.* (1990), which is around 59 000 Daltons.

The protein sequence predicted from the DNA sequence shows regions with high similarity to the yeast homolog (Fig. 24). As with the *E. coli* protein sequence this similarity is restricted to the amino terminal half of the protein (Fig. 24). Some of the regions of identity/similarity between yeast and *E.coli* are also seen between the yeast and lupin protein sequences (Fig.25). It is possible that these conserved regions play an important role in the structure or function of

FIGURE 24: Protein sequence identity/similarity between yeast and lupin tRNA nucleotidyltransferase. L and Y on the left margin correspond to Lupin and Yeast respectively.

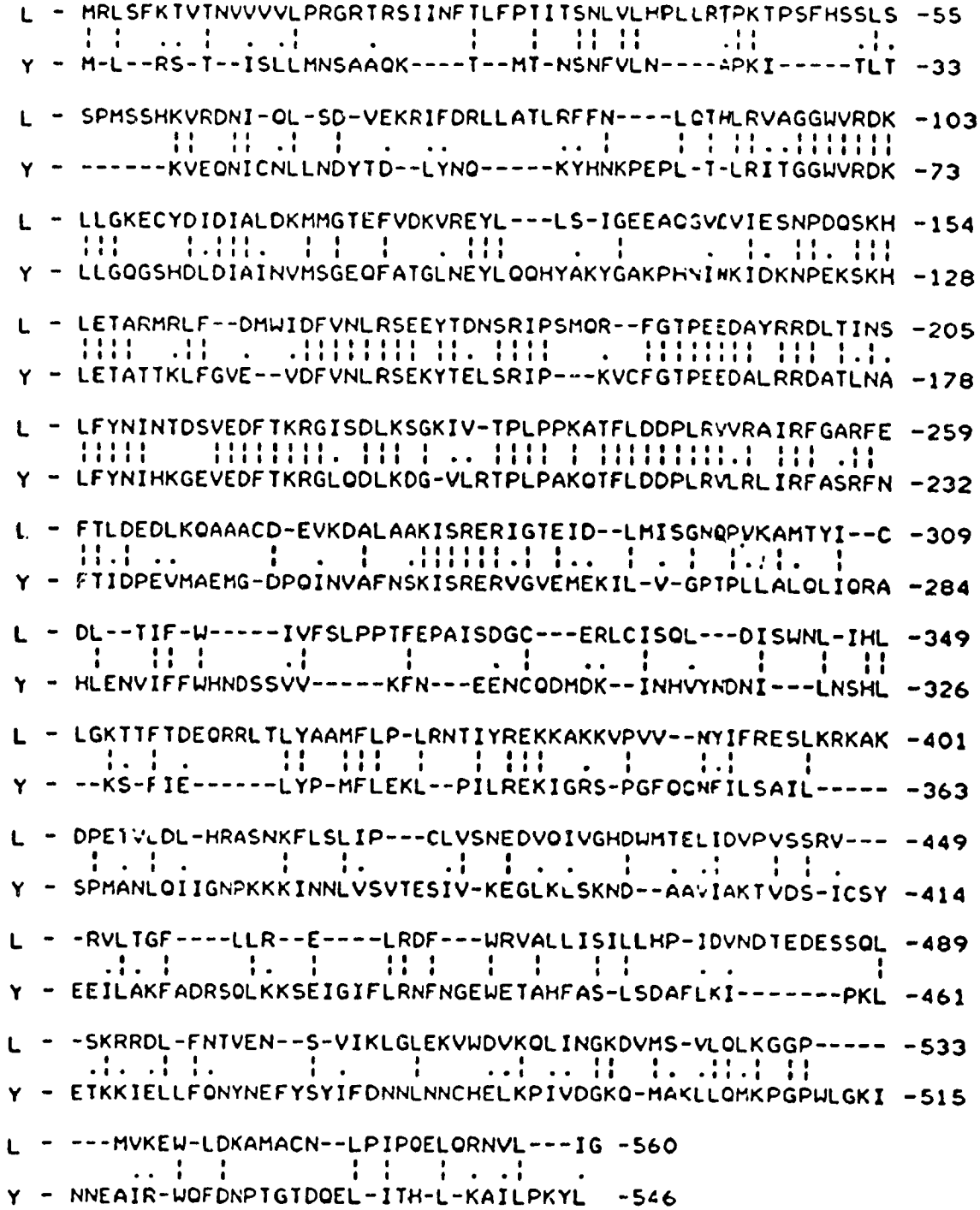


Fig. 25 Protein sequence identity/similarity among lupin, yeast and *E.coli* tRNA nucleotidyltransferase

```
LUPIN     189  F  G  T  P  E  193
               |  |  |  |  |
YEAST     162  F  G  T  P  E  166
               "  "  "  |  "
E.coli     78  Y  A  A  P  D   82
```

```
LUPIN     243  D  D  P  L  R  V  V  R  A  I  R  F  G  A  R  F  258
               |  |  |  |  |  |  "  |  "  |  |  |  "  "  |  |
YEAST     226  D  D  P  L  R  V  L  R  L  I  R  F  A  S  R  F  231
               "  |  |  |  |  |  |  |  "  "  |  |  |  "  |  "
E.coli    131  E  D  P  L  R  V  L  R  V  A  R  F  A  A  R  V  146
```

```
LUPIN     282  K  I  S  R  E  R  I  288
               |  |  |  |  |  |  "
YEAST     255  K  I  S  R  E  R  V  262
               "     "  "  |  |  |
E.coli    172  G  -  L  P  E  R  V  178
```

The character to show that two aligned residues are identical is '|' and the character to show that two aligned residues are similar is ' " '.

this protein. The predicted ATP binding domain from the *E. coli* enzyme, (Cudny *et al.*, 1986) appears to be absent from both the yeast and lupin enzymes.

Since this protein was isolated from crude extract it is most likely that this protein represents the nucleocytoplasmic form of this enzyme which would be expressed from a nuclear gene. Although there is as yet no definite consensus sequence for nuclear localisation signals most of the proteins targeted to the eukaryotic nucleus contain the sequence Lys-Arg/Lys-X-Arg/Lys (Chelsky et al., 1989). These 4 residues are seen twice in the predicted amino acid sequence of lupin tRNA nucleotidyltransferase (Underlined in Fig.23) suggesting that this protein may contain a nuclear localisation signal.

4) CONCLUSIONS AND FUTURE WORK

In the process of cloning a full-length cDNA for tRNA nucleotidyltransferase the protein was purified, characterised and 2 individual peptide fragments sequenced. This information was used to isolate a cDNA clone that encoded tRNA nucleotidyltransferase. This represents the first tRNA nucleotidyltransferase cDNA to be characterised in any multicellular eukaryote.

We are confident that the clone we have isolated codes for the *Lupinus albus* tRNA nucleotidyltransferase. The purified protein shows characteristics that are similar to those of other tRNA nucleotidyltransferases including the enzyme previously isolated from the related species *L. luteus*. The sequence of this gene resembles that of the only other eukaryotic tRNA nucleotidyltransferase

gene that has been isolated (*S. cerevisiae*). Our cDNA can complement a temperature sensitive mutation in this gene in *S. cerevisiae* indicating that we have a cDNA encoding a functional tRNA nucleotidyltransferase.

Our major interest in isolating this gene was to determine whether or not this gene could encode multiple products which might function in different subcellular locations, *i.e.*, are the nucleocytoplasmic and the mitochondrial forms of tRNA nucleotidyltransferase encoded by the same gene as in yeast. Preliminary analysis of the sequence of this gene suggests two possible motifs that could represent nuclear targeting signals, but no apparent mitochondrial or chloroplast targeting information. The open reading frame of our longest cDNA clone, however, extends beyond the length of the cDNA we have so that it is possible that we do not have a full-length cDNA and that a mitochondrial or chloroplast targeting signal may be encoded further upstream in this gene. The fact that the sequence around the first in frame start codon in this sequence does not resemble a typical plant start codon may also suggest that we have not cloned a full length cDNA. However, we have also cloned a second cDNA that shows a remarkable degree of similarity with our first clone except that it does contain a stop codon 6 bases upstream of the first in frame start codon. The high degree of similarity between these clones in their coding sequences and in their 5' and 3' flanking sequences suggests that they are products of the same gene and that any differences that are found are the result of artifacts created in the cloning and sequencing process. If the second cDNA represents the real genomic sequence

then this indicates that we have cloned a full-length cDNA coding for lupin tRNA nucleotidyltransferase and that there are no additional 5' sequences which may code for additional targeting information.

R. Roy (personal communication) has shown that under stringent hybridisation conditions . single signal is seen in genomic restriction digestions of lupin DNA probed with this gene suggesting that this gene is present as a single copy. It would be interesting to clone this gene to show which of our cDNAs represent transcripts of the actual gene and to study regions upstream of this coding sequence that might be responsible for its regulation. Differential transcription patterns have been shown to be responsible for producing proteins targeted to multiple intracellular locations in yeast (Ellis *et al.*, 1987) and it would be interesting to see if this is also the case in lupin. Northern hybridisation and primer extension may also help to answer this question. Since a single gene is evident on Southern hybridisation this suggests two major possibilities. Either this gene is responsible for producing the tRNA nucleotidyltransferase that is targeted to all locations in the plant cell, or there are other genes present with a significant difference in sequence. Earlier hybridisation results (Cudny *et al.*, 1986) and our own experience with heterologous hybridisation suggest that although tRNA nucleotidyltransferases share some amino acid identity they lack significant similarity at the nucleotide level. Therefore, there may be other nuclear tRNA nucleotidyltransferase genes that were not localized because of differences in

DNA sequence. Perhaps hybridisation under lower stringency conditions will reveal these genes.

While these hybridisation experiments are ongoing we can attempt to identify potential targeting signals in the tRNA nucleotidyltransferase coded by the cDNA we have sequenced. Because this is the major form of tRNA nucleotidyltransferase isolated from lupin it probably represents the nucleocytoplasmic form of the enzyme and therefore should contain nuclear targeting information. We have identified two potential nuclear localization signals and experiments can now be initiated to determine whether these amino acids are necessary and sufficient for nuclear localisation. Because tRNA nucleotidyltransferase is an essential gene in eukaryotes these experiments have to be conducted using gene fusions.

Finally, we can now isolate tRNA nucleotidyltransferase from other intracellular compartments to compare it at the enzyme level and eventually at the gene level to the tRNA nucleotidyltransferase characterised in this study to determine definitively whether they are products of the same or different genes.

Table 2

APPENDIX A

EFFECT OF DIFFERENT INCUBATION TIMES ON THE ACTIVITY OF tRNA NUCLEOTIDYLTRANSFERASE

TIME (MINUTES)	ACTIVITY (cpm)		
	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3
0			173 286 539
10	225 254	2768 4151	4263 4094 4241
20	952	5403 3339	5309 5352 5121
30			5952 5536 5097
40	3406 1292	10911 9577	5562 5284 5941
50	1565 1962	16465 11124	
60	1618 1871	11565 10405	5200 5537 6162
70	3292 3908	15740 12786	

Table 3

APPENDIX B

EFFECT OF DIFFERING CONCENTRATIONS OF GLYCINE ON THE ACTIVITY OF tRNA NUCLEOTIDYLTRANSFERASE

[GLYCINE]	ACTIVITY (cpm)*	
	EXPERIMENT 1	EXPERIMENT 2
10 mM	533 576	3120 3285
50 mM	692 753	3050 4609
100 mM	780 724	4991 5050
150 mM	798 749	4882 4830
200 mM	575 801	5550 5004
250 mM	731 746	5149 4721
300 mM	808 847	5504 5439
350 mM	645 864	5615 5360
400 mM	832 702	5949 5948

* These data represent the 30 minute time point in each case.

Table 4

APPENDIX C

EFFECT OF DIFFERENT pHs ON THE ACTIVITY OF

tRNA NUCLEOTIDYLTRANSFERASE

pH	ACTIVITY (cpm)*	
	EXPERIMENT 1	EXPERIMENT 2
7.5	725 503	1033 940 1009
8	2364 1284	1346 1349 1659
8.5	1886 1828	2247 2555 2201
9	2973 3000	2621 1844 2401
9.5		1933 2031 2012
10	1733 2341	1757 1659 1905

* These data represent the 30 minute time point for experiment 1 and the 20 minute time point for experiment 2.

APPENDIX D

Table 5

EFFECT OF DIFFERING CONCENTRATIONS OF CTP ON THE INCORPORATION OF [α^{32}P] ATP BY tRNA NUCLEOTIDYLTRANSFERASE

[CTP]	ACTIVITY (cpm)							
	EXPERIMENT 1				EXPERIMENT 2			EXPERIMENT 3
	TIME (MINUTES)				TIME (MINUTES)			TIME (MINUTES)
	10	20	40	60	10	20	40	20
0 mM								734 766 884
0.01 mM	5516 5222	7604 8496	8401 8550	9692 9819	404 447	367 793	571	1934 1724 2727
0.1 mM	5632 5243	7942 7653	9139 9310	9579 9412	248 250	237 320	351 341	2256 2465 3347
0.2 mM	4484 4024	6843 6093	7486 7719	9287 8483	201 279	295 225	560 553	1747 1912 2452
1 mM	1919 1575	2648 2984	3167 3360	4457 4373	109 236	114 134	194 186	900 781 751
10 mM	153 27	578	162	73 173	1		1 69	174 213 669

TABLE 6: Effect of differing concentrations of ATP on the incorporation of [α^{32}P] ATP by tRNA nucleotidyltransferase

Appendix E

[ATP]	ACTIVITY (Cpm)								
	EXPERIMENT 1				EXPERIMENT 2				EXPERIMENT 3
	TIME (MINUTES)				TIME (MINUTES)				TIME (MINUTES)
	10	20	40	60	10	20	40	60	20
0 mM									9611 9940 10272
0.01 mM	17710 14622	37121 24220	47806 35798	64806 51758	223 206	210 287	378 320	379 353	8417 8441 8996
0.1 mM	9174 7968	13983 16392	14932 14273	17134 19151	152 218	676 312	318 382	469 483	5686 5439 5771
0.2 mM	8153 6275	12769 8654	8589 9796	9778 10039	77	52 65	208 149	386 223	4007 3921 3414
1 mM	1998 2576	2509 4225	2038 8915	2021 3872	11 83	126 101	85 76	103 164	1039 1049 1602
10 mM	1368	198	751 4225	3405 4462	15 6	30 90	125 74	67 65	10 358 297

Table 7

APPENDIX F

EFFECT OF TEMPERATURE ON THE ACTIVITY OF

tRNA NUCLEOTIDYLTRANSFERASE

TEMPERATURE (°C)	ACTIVITY (cpm)					
	EXPERIMENT 1			EXPERIMENT 2		
	TIME (MINUTES)			TIME (MINUTES)		
	20	40	60	20	40	60
10	117 92	133	5 267	965 1443	2007	3579 2870
RT	155 216	486 363	603 412	1896 2633	5651 2886	4355 7455
30	436	725	1115	2989 3217	3855 4402	4589 4028
37	320 571	695 856	1201 1338	4505 4435	7949 6249	6480 6091
45	527 597	1060 1012	1480 1681	5168 5406	5723 6130	7031 7258
65				1463 1507	1786 1505	1298 1229

APPENDIX H

EFFECT OF [KCl] ON THE ACTIVITY OF tRNA NUCLEOTIDYLTRANSFERASE

[KCl]	ACTIVITY (Cpm)				
	EXPERIMENT 1			EXPERIMENT 2	EXPERIMENT 3
	TIME (MINUTES)			TIME (MINUTES)	TIME (MINUTES)
	20	40	60	60	20
0 mM	6126 6354	7905 8250	8490 8418	38446 33150	4361 4263 4567
100 mM	7566 7312	9403 9591	1031 964	32606 32446	4401 4513 4832
200 mM	6928 7209	8648 9117	8720 9177	32311 33355	4335 4789 4784
400 mM	4494 4373	6963 7018	6963 7018	31168 32876	3503 2500 2447
600 mM	1538 1844	3503 3788	3503 3788	29389 28037	1871 1270 973

Table 9

APPENDIX G

EFFECT OF [NaCl] ON THE ACTIVITY OF tRNA NUCLEOTIDYLTRANSFERASE

[NaCl]	ACTIVITY (Cpm)				
	EXPERIMENT 1			EXPERIMENT 2	EXPERIMENT 3
	TIME (MINUTES)			TIME (MINUTES)	TIME (MINUTES)
	20	40	60	60	20
0 mM	5696 5373	6781 7203	7849 8861	35516 30895	5222 5220 6020
100 mM	7126 6629	8097 7294	8097 7391	34553 34282	5236 5359 5198
200 mM	5405 6124	6614 6884	7087 7154	33316 37784	4255 4111 4115
400 mM	2290 2790	4940 4244	4940 4244	32316 31151	734 1497 1415
600 mM	1157 8326	2614 1783	3757 2844	26452 27416	372 247 1300

Table 10

APPENDIX I

EFFECT OF $MgCl_2$ ON THE ACTIVITY OF tRNA NUCLEOTIDYLTRANSFERASE

$[MgCl_2]$	ACTIVITY (cpm)			
	EXPERIMENT 1			EXPERIMENT 2
	TIME (MINUTES)			TIME (MINUTES)
	20	40	60	20
0 mM	335	395	1040 1412	690 1691 383
0.1 mM	1891 1693	3106 2409	4511 3841	931 1117 931
1 mM	2544 3121	4341 4688	5976 5716	2668 2796 4167
10 mM	4174 3728	5033 4620	6893 7000	3548 5920 2916
100 mM	1176 2750	2466 4180	4994 6360	2323 1474 1729

Table 11

APPENDIX J

EFFECT OF $MnCl_2$ ON THE ACTIVITY OF tRNA NUCLEOTIDYLTRANSFERASE

[$MnCl_2$]	ACTIVITY (cpm)			
	EXPERIMENT 1			EXPERIMENT 2
	TIME (MINUTES)			TIME (MINUTES)
	20	40	60	20
0 mM	328 318	524 496	2232 1455	1391 1331 928
0.1 mM	1648 1602	3011 2670	5448 5934	1493 1528 436
1 mM	1508 1536	2519 2237	4450 4808	2359 2699 2740
10 mM	1434 1602	2689 2874	3103 3888	2067 2070 2738
100 mM	207	1093 825	1244 2312	977 1397 1057

Table 12

APPENDIX K

EFFECT OF [EDTA] ON THE ACTIVITY OF

tRNA NUCLEOTIDYLTRANSFERASE

[EDTA]	ACTIVITY (cpm)			
	EXPERIMENT 1			EXPERIMENT 2
	TIME (MINUTES)			TIME (MINUTES)
	20	40	60	20
0 mM	4944 5592	9801 8807	8198 8036	4194 4900 3795
0.5 mM	4175 3870	5842 5821	8667	3765 3353 3822
1 mM	3163 3287	4792 5110	6749 6503	3750 3827 3858
10 mM	0 0	20 50	150	670 1026 1653

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