Investigation of messenger RNA stability in the yeast *Saccharomyces cerevisiae*

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by

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Dedicated to my parents

and Annie and Sabina

for all their love and kindness.

CONTENTS

ABBREVIATIONS

Chemicals

ATP - adenosine triphosphate

BSA - bovine serum albumin

dATP - deoxyadenosine triphosphate

dCTP - deoxycytidine triphosphate

dGTP - deoxyguanosine triphosphate

dTTP - deoxythymidine triphosphate

DNA - deoxyribonucleic acid

cDNA - complementary DNA

cDNAn - plasmid DNA from nth cDNA clone

ssDNA - single-stranded DNA

dsDNA - double-stranded DNA

RNA - ribonucleic acid

mRNA - messenger RNA

mRNAn - mRNA complementary to cDNAn

DEPC - diethylpyrocarbonate

DTT - dithiothreitol

EDTA - ethylenediaminetetra-acetic acid (disodium salt)

PAGE - polyacrylamide gel electrophoresis

SDS - sodium dodecylsulphate

SSC - saline sodium citrate

TBE - tris-borate, EDTA

TCA - trichloroacetic acid

TE - tris, EDTA

Tris - tris (hydroxymethyl) amino ethane

Measurements

V - volts

Kd - kilo Dalton

bp - base pair

Kb - kilo base pair(s)

^{o}C -degrees centigrade

g - centrifugal force equal to gravitational acceleration

cm - centimetre (10^{-2}m)

mm - millimetre (10^{-3}m)

g - gramme

mg - milligramme (10^{-3}g)

ug - microgramme (10^{-6}g)

ng - nanogramme (10^{-9}g)

l - litre

ml - millilitre (10^{-3} l)

ul - microlitre (10^{-6} l)

mw - molecular weight

w/v - weight/volume

v/v - volume/volume

M - molar (moles per litre)

mM - millimolar (10^{-3}M)

uM - micromolar (10^{-6}M)

Ci - Curie

uCi - microCurie (10^{-6} Ci)

pH - acidity [negative $\log_{10}$ (molar concentration H^{+} ions)]

Miscellaneous

log - logarithm

UV - ultra violet light

Fig. - figure

cpm - counts per minute

rpm - revolutions per minute

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SUMMARY

Factors which influence mRNA stability in the yeast, Saccharomyces cerevisiae have been investigated, in particular the influence of mRNA length and translation. The properties of 13 mRNAs of unknown function have been analysed using yeast cDNA clones as molecular probes. The pyruvate kinase mRNA was also analysed using a genomic clone.

Preliminary characterization of each cDNA clone by northern blotting, Southern blotting and cross hybridization experiments demonstrated that each was derived from a different, single copy gene (with the possible exception of cDNA10).

A new and convenient technique has been developed for the measurement of mRNA half-life in yeast. The method involves the quantitation of specific mRNAs by dot-blotting after inhibition of transcription using phenanthroline. The chemical half-lives for the 14 mRNAs ranged from 6.6 $\pm$ 0.67 to over 100 minutes, relative to the half-life of the 18S rRNA control.

Comparison of mRNA length and half-life revealed that two populations of mRNAs containing relatively stable or unstable mRNAs were present. Within each population there was an inverse relationship between mRNA length and half-life. The distribution of each mRNA across sucrose density gradients of yeast polysomes was analysed. There was no obvious correlation between 'ribosome loading' and mRNA half-life. Therefore mRNA length, but not

ribosome loading, would appear to be an important factor in the determination of the stability of an mRNA in S.cerevisiae. To account for the division of yeast mRNAs into two clear populations on the basis of mRNA length and half-life, it is suggested that at least one other factor must have an influence.

CHAPTER 1

INTRODUCTION

Introduction

The steady-state level of a specific eukaryotic mRNA is dependant upon the rates of transcription, mRNA processing, transport to the cytoplasm and degradation of the appropriate sequences. The stabilities of eukaryotic mRNAs show great diversity even within the same organism. For example, human beta and delta-globin mRNAs have a half-life of 16.5 and 4.5 hours, respectively (Ross and Pizarro, 1983). Also, actin and tubulin mRNAs have half-lives of 16-26 and 7 hours, respectively in mouse L cells (Krowczynska et al., 1985). In Saccharomyces cerevisiae mRNA half-lives range from about 3.5 minutes to more than 70 minutes for individual mRNAs (Chia and McLaughlin,1979; Koch and Friesen, 1979).

A number of factors have been reported to affect mRNA stability. External factors such as hormones, viral infection and cellular differentiation have been shown to affect the half-lives of specific mRNAs. At the molecular level, structural features of an mRNA such as 3'-poly(A) sequences, the 5'-cap, internal nucleotide sequences and mRNA length are intimately associated with mRNA half-life.

As early as 1974 hormones were reported to influence the stability of specific mRNAs. For example, Palmiter and Carey (1974) showed that avian ovalbumin mRNAs were stabilized by administering oestrogen, whereas the withdrawal of oestrogen reduced the stability of the ovalbumin mRNA by ten fold. Guyette et al. (1979) showed that prolactin, a peptide hormone,

stabilized the casein mRNA by 17-25 fold in mammary gland cultures. Also, oestrogen selectively stabilized Xenopus laevis liver vitellogenin mRNA against cytoplasmic degradation (Brock and Shapiro,1983). Vitellogenin mRNA showed a half-life of approximately 500 hours in the presence of oestrogen, which decayed with a half-life of 16 hours in the absence of oestrogen. However, the presence or absence of oestrogen did not alter the rate of decay for the poly(A)+ mRNA population as a whole. Diamond and Goodman (1985) showed that growth hormone mRNA was stabilized in rat pituitary tumour cells by dexamethasone and by the thyroid hormone, L-triiodothyronine. They reported a half-life of 20 hours for growth hormone mRNA in the tumour cells under hormone induction. In the absence of hormone, the half-life of the growth hormone mRNA was reduced to 2 hours.

Though these results show that hormones influence the stability of a specific set of mRNAs, the mechanism by which this is brought about is unclear. Palmiter and Carey (1974) have suggested that certain RNases could be synthesised more rapidly or activated in the absence of hormones to bring about increased rates of degradation. Alternatively, specialized proteins might stabilize specific mRNAs against RNases by maintaining a certain conformation or by holding the mRNAs in a protected environment (Palmiter and Carey, 1974).

Cellular development also has some influence on the stability of certain mRNAs. Anderson and Lengyel (1980) showed that in Drosophila embryos, histone mRNAs turned over at different rates depending on the stage of cell differentiation.

The half-life of the histone mRNAs decreased from 2.3 hours at the blastoderm stage, to 20 minutes by the end of gastrulation. Also, Howe et al.(1984) have reported that the alpha-tubulin mRNAs and an mRNA encoding a 94000 Dalton protein (p94) decayed at faster rates in a differentiated teratocarcinoma derived parietal endoderm cell line (PYS-2) compared with an undifferentiated teratocarcinoma stem cell line (F9). The half-lives of both alpha-tubulin and p94 mRNAs were greater than 9 hours in undifferentiated F9 cells compared to 2 hours for alpha tubulin mRNA and 5 hours for p94 mRNA in differentiated PYS-2 cells.

Viral infections can also bring about variations in the metabolism of mRNAs in the host cell. Nishioka and Silverstein (1977) observed a rapid degradation of globin mRNA during infection by herpes simplex virus (HSV). When friend erythroleukemia (FL) cells were infected with HSV,there was rapid inhibition of globin synthesis, followed by a rapid degradation of globin mRNA. On the other hand, infection of FL cells by Vesicular Stomatitis Virus (VSV) neither brought about the inhibition of protein synthesis nor a discernable change in the half-life of the globin mRNA (Nishioka and Silverstein 1978). Rice and Roberts (1983) studied the degradation of alpha-tubulin and beta-actin mRNAs in mouse L cells during infection with Vaccinia virus. The half-life of both mRNAs were more than 6 hours in normal mouse L cells, but after infection with Vaccinia virus, the alpha-tubulin and beta-actin mRNA half-lives were reduced to 3 hours. In contrast, Khalili and Weinmann (1984)

reported that actin mRNA was stabilized in Adenovirus infected HeLa cells compared to the uninfected cell line. Uninfected HeLa cells showed a half-life of less than 6-12 hours for actin mRNA whereas the Adenovirus infected cells showed a half-life of more than 14-24 hours for actin mRNA. This stabilization is possibly brought about by the inactivation of existing cellular RNases, or by the synthesis of cellular or viral factors that bind to mRNAs and protect them from degradative enzymes.

The Vaccinia virion is known to contain two single-strand specific DNases localized in the viral core (Pogo and Dales, 1969). It is uncertain whether these DNases have any RNase activity or if any other RNase is present within the viral core. HSV infections induce morphological changes in infected cells (Falke et al., 1985). These morphological changes could cause recompartmentalization of mRNAs within the cell and hence cause changes in mRNA turnover. Poliovirus has been shown to cause relocalization of mRNA within the cell. mRNA becomes detached from the cytoskeleton and enters the soluble cytoplasmic compartment of the infected HeLa cell (Lenk and Penman, 1979). However, mRNA half-life does not appear to change during poliovirus infection.

Little is known about the exact mechanism by which mRNA stabilization or destabilization is brought about, during adenovirus, vaccinia or HSV infection. However,there is good evidence for the activation of latent cellular RNases by interferon. In interferon treated cells, a series of 2'-5' linked oligoadenylic acid triphosphates (2'-5'A) are synthesised.

These have been shown to destabilize mRNAs by activating a latent RNase (RNase L; Baglioni et al., 1978). Wreschner et al.(1981) and Smith et al.(1981) have studied the RNA cleavage pattern mediated by the RNase-L. End-labelled (3'or 5') influenza virus RNAs 7 and 8 were digested in the presence of 2'-5'A with RNase-L from the rabbit reticulocyte, mouse ascites tumour cell or human lymphoblastoid cell lysate. Their results showed that though RNase-L specifically cleaved at UpN sequences, UA and UU sequences were preferred. The enzyme from the rabbit reticulocyte lysate cleaved at UA sequences, particularly within sequences rich in U residues. However, the preference for sites in a U-rich region was not obvious with the enzymes from the human and mouse cells. Therefore, RNase-L from different sources shows similar, but not identical specificities. They also reported that not all UA or UU sequences were cleaved. It was suggested that this might be due to secondary structure formation which might protect some potential cleavage sites. These authors also demonstrated that this cleavage is not unique to viral RNA, since rRNA was also cleaved at UpNp sequences. Using synthetic homopolymers, Smith et al. (1981) have shown that RNase-L from Ehrlich ascites tumour cells cleaves only poly(U) and not poly(A), poly(C) and poly(G). They tested the cleavage preference further, using end-labelled T7 RNA segments. The cleavage occurs at single stranded UA,UU and UG sequences only. With the widespread existence of the enzyme 2'-5'A synthetase and RNase-L (Stark et al.,1979) it is tempting

to speculate that these enzymes may play a role in the normal catabolism of RNA and that they might be activated by factors other than interferon or hormones. However, this must be proven experimentally.

Growth conditions have been shown to alter mRNA half-lives in various organisms. In E.coli, the stability of the 'ompA' and 'CAT' mRNAs demonstrates a marked dependence on cell growth rate (Nilsson et al., 1984). These authors found that the 'ompA' mRNA, whose half-life was 15 minutes when E.coli was grown in L-broth (doubling time 40 minutes),was reduced to 4 minutes in cells grown in MOPS/Acetate medium (doubling time 200 minutes). Similarly the 'CAT' mRNA in L-broth had a 2 minute half-life, and this was reduced to 0.4 minutes in MOPS/Acetate medium. However, 'Lpp' and 'bla' mRNAs which had half-lives of 22 and 3 minutes in L-broth, respectively, did not change in the MOPS/acetate medium. These results suggest that the decay rates of specific mRNAs are affected differently by changes in the rates of cell growth. In S.cerevisiae, a decrease in growth rate resulted in a increase in the half-life of the allophanate hydrolase mRNA (Cooper et al., 1978). This is in contrast to the observation in E.coli where an increase in the doubling time reduced the half-life.

In addition to the external factors described above, mRNA half-lives are influenced by the structural features of the mRNA molecule itself. Structures such as the 3'-poly(A) tail, the 5'-cap, sequence signals and mRNA length have been shown to influence mRNA stability.

The 3'-poly(A) tract, present on most eukaryotic mRNAs, influences mRNA stability (Huez et al., 1974; Huez et al., 1975; Nudel et al., 1976). Huez et al. (1974) reported that native rabbit globin mRNA is very stable and is translated very efficiently for over 70 hours after injection in to Xenopus oocytes. In contrast, deadenylated globin mRNA was found to have a very short half-life of 5-10 hours. Similar results were obtained when native and deadenylated rabbit globin mRNAs were injected into HeLa cells (Huez et al., 1981), showing that the 3'-poly(A) tract has a stabilizing role in different systems. However, no differences in stability were observed between alpha-2-u globulin rat liver mRNAs with average poly(A) tail lengths of 175 and 40 nucleotides (Deshpande et al., 1979). The hypothesis that the 3'-poly(A) tract stabilizes the mRNA, was critically tested by enzymatic addition of a poly(A) segment to unstable deadenylated globin mRNA. This resulted in the complete restoration of the functional and physical stability of rabbit globin mRNAs in frog oocytes (Huez et al., 1975). The stabilizing role of the poly(A) tail was further confirmed using HeLa cell histone mRNAs,which lack 3'-poly(A) residues. The histone mRNA was enzymatically polyadenylated, injected into Xenopus oocytes and its functional stability examined by measuring the kinetics of histone synthesis. It was also found that enzymic polyadenylation increased the stability of the histone mRNA under these conditions (Littauer and Soreq 1982).

Attempts have been made to correlate the length of the 3'-poly(A) tail with the mRNA stability. Globin mRNA preparations containing poly(A) segments of decreasing length were prepared by partial phosphorolysis with polynucleotide phosphorylase (Nudel et al., 1976). When injected into Xenopus laevis oocytes, globin mRNA species containing 30 or more adenylate residues were as stable as the native globin mRNAs, whereas those having 16 adenylate residues were unstable as deadenylated globin mRNAs (Nudel et al. 1976). Thus there would appear to be no change in mRNA half-life until the poly(A) tail is shortened below a critical length. These results are in agreement with the report of Carlin (1978) who showed that there was an increase in mRNA half-life up to a length of 30 adenylate residues per mRNA chain, after which there was no obvious correlation between poly(A) tail length and half-life.

The exact mechanism by which this stabilization is brought about is not clear. The simplest explanation is that poly(A) sequences protect the mRNA from 3'-exonucleolytic degradation. Blobel (1973) has isolated a protein which has a great affinity for poly(A) sequences either at the 3'-end of an mRNA or at other sites (the poly(A) binding protein). By virtue of its ability to bind to the 3'-poly(A) tail it may possibly protect the mRNA from 3'-exonucleolytic degradation.

Some investigators did not find such a stabilizing role by the 3'-poly(A) tail. For example, Palatnik et al. (1980) have shown that there was no correlation between poly(A) length and mRNA longevity in Dictyostelium discoidium. Similarly, the

stability of the human fibroblast interferon mRNA in microinjected Xenopus oocytes was unaffected by the removal of the poly(A) tail (Seghal et al., 1978). Similar results were obtained for mRNAs coding for two species of interferons in human fibroblasts (Seghal and Sagar, 1980; Weissenbach et al., 1980).

All cellular eukaryotic mRNAs studied so far contain a blocked methylated structure termed a 'cap' at their 5'-termini. (Some viral mRNAs do not have a 5'-cap). It was suggested that this 5'-cap might play a role in stabilizing eukaryotic mRNAs by conferring resistance to 5'-exonucleolytic degradation. Furuichi et al.(1977) showed that the 5'-cap of reovirus mRNA has a stabilizing effect whether the mRNA is injected into Xenopus oocytes, or added to wheat germ or mouse L-cell protein synthesising extracts. In all these three systems, reovirus mRNAs containing either a 5'-methylated cap or 5'-unmethylated cap were degraded more slowly than molecules without the cap. There was no difference in stability between mRNA molecules possessing methylated or unmethylated caps. However, mRNA molecules with unmethylated caps were not translated efficiently. This suggested that the stabilization of the capped mRNA molecules was not due to their participation in protein synthesis, but rather to an intrinsic property of RNAs with 5'-caps. Shimotohno et al. (1977) observed a preferential degradation of uncapped polyhedrosis virus (CPV) mRNAs in wheat germ extracts.

Furuichi et al. (1977) have analysed the mechanism by which the 5'-cap protects an mRNA from degradation. Uncapped, 5'-end-labelled mRNA was injected into the Xenopus oocyte or added to a wheat germ translational system. After incubation, the radioisotope was present either in full length mRNA or as 5'-mononucleotides; there was no radioactivity observed in products of intermediate length. This suggested that the 5'-cap protects mRNA from 5'-exonucleolytic degradation. However, uncapped poliovirus RNAs in poliovirus are stable and are translatable in various in vitro systems (Rose et al., 1978). It is possible that these uncapped molecules may be protected against exonucleolytic degradation by interaction with proteins or by maintaining a specific conformation at their 5'-termini (Furuichi et al., 1977).

The 3'- or 5'-untranslated regions of some mRNAs have been implicated in mRNA stability. The 5'-untranslated region has been shown to play a role in stabilizing some viral (Gorski et al.,1985) bacterial (von Gabain et al., 1983; Green and Inouye, 1984) and eukaryotic (Eick et al., 1985) mRNAs. The mechanism by which this occurs is not clear. However, these authors suggested that secondary structure formation in this region may bring about this stabilization.

The difference in the half-lives of human beta- and delta-globin mRNAs (16.5 and 4.5 hours respectively) has been attributed to sequence divergence in the 3'-untranslated region. The two mRNAs show about 90% sequence homology in the 5'-untranslated region and coding region whereas they are only about

40% homologous in the 3'-untranslated region (Ross and Pizarro, 1983). Yaffe et al. (1985) compared the sequences of mRNAs coding for several vertebrate actins and found a high degree of sequence homology in the 3'-untranslated region. Sequence conservation was also found in the 3'-untranslated regions of other genes such as mouse and human beta-nerve growth factors (Yaffe et al., 1985). The authors also found that the length of the 3'-untranslated region of mRNAs coding for similar types of protein in distantly related organisms are often similar. This conservation may play a role at the transcriptional or post-transcriptional level.

Caput et al.(1986) have observed a conserved domain comprised entirely of A and U residues in the 3'-untranslated regions of the murine and human tumour necrosis factor (TNF) mRNAs. The consensus sequence UUAUUUAU was present within this domain. The same sequence was also found in mRNAs encoding human lymphotoxin,human colony stimulating factor, human and mouse interleukin 1, human and rat fibronectin and most of the sequenced human and mouse interferons. Shaw and Kamen (1986) have introduced a synthetic 51 nucleotide AT sequence, which is present in the human lymphokine gene, into the 3'-untranslated region of the rabbit beta-globin gene. This caused the otherwise stable beta-globin mRNA to become highly unstable in vivo. They have proposed that the A:U rich sequence is the recognition signal for an mRNA processing pathway which specifically degrades the mRNAs for certain lymphokines,cytokines and proto-oncogenes. The pathway might involve RNase L (which is induced by 2'-5'A, as described above) since this enzyme is specific for A:U-rich

sequences (Smith et al., 1981; Wreschner et al., 1981).

As early as 1973 Singer and Penman suggested a correlation between mRNA length and half-life. Pulse/chase labelling experiments revealed the existence of stable and unstable populations within HeLa cell mRNA, with average half-lives of 24 and 7 hours, respectively. A small but significant difference in the sedimentation profile of stable and unstable mRNAs was observed on sucrose gradients. The unstable mRNA population was larger on average than the stable mRNA population. Similar results were obtained with a mosquito cell line by Spradling et al. (1975). The difference in length between the stable and unstable population was much more pronounced than in HeLa cells. These authors showed that this difference in length, as revealed by sedimentation analysis, was neither an artefact nor due to cleavage of long molecules into shorter molecules. Similar observations have been made in resting human lymphocytes by Berger and Cooper (1975) and in mouse L cells by Meyuhas and Perry (1979). This relationship between length and stability suggests that longer mRNAs might contain more nuclease sensitive sites than shorter mRNAs and thus might be more vulnerable to random degradative events. However, no direct relationship between mRNA length and stability could be made using these gross analyses of mRNA populations, since large overlaps were observed between stable and unstable populations. For example, some relatively long mRNAs were present in the stable mRNA populations, and some relatively short mRNAs were observed in the unstable mRNA populations.

Blundell et al. (1972) have shown that there is no correlation between mRNA length and half-life in E.coli, by comparing the half-lives and lengths for individual mRNAs. However, the mRNAs were polycistronic and only six mRNAs were analysed in their study.

The possibility that mRNA stability may be correlated with its abundance was suggested by Bishop et al.(1974). Obviously differences in the relative abundance of mRNA species may reflect differences in their rates of synthesis, or degradation, or both. Lenk et al.(1978) studied the relationship between mRNA abundance and stability in Drosophila and HeLa cell lines. In Drosophila cells abundant mRNAs tended to be long-lived and low-abundance mRNAs were short-lived. However, in HeLa cells they did not find such a correlation. Another group (Meyuhas and Perry, 1979) have found a correlation between mRNA abundance and half-life in mouse L cells. Therefore, it would appear that turnover rate is more important in determining mRNA abundance in some organisms than others.

A relationship between translation and mRNA stability has been reported in E.coli (Morse and Yanofsky, 1969; Morse et al., 1969). They observed that mRNAs carrying premature stop codons were less stable than their wild-type counterparts. Losson and Lacroute (1979) and Pelsy and Lacroute (1984) also made similar observations in S.cerevisiae. These observations are discussed in more detail in Chapter 6.

In this study an attempt has been made to investigate the contentious issue of whether the length of an mRNA is related to its half-life in S.cerevisiae. All previous studies involving eukaryotic systems have analysed gross mRNA populations on sucrose gradients (Singer and Penman, 1973; Spradling et al., 1975; Berger and Cooper, 1975; Meyuhas and Perry, 1979), and could not account for the existence of long mRNAs in stable populations or short mRNAs in unstable populations. An alternative way of investigating the correlation between mRNA length and half-life would be to analyse the properties of a number of specific mRNA species. The approach has been to construct and purify cDNA clones for use as probes for specific mRNAs. Sequences of unknown function have been used in an attempt to prevent bias in the analysis which might arise through the analysis of mRNAs of known function (e.g. glycolytic mRNAs). A new method has been developed for the measurement of mRNA half-life.

An attempt has also been made to investigate the correlation between mRNA stability and translation. The cDNA probes have been used to determine the distribution of specific mRNAs on sucrose density gradients of yeast polysomes. The polysome distributions have been compared with the half-lives of the mRNAs in question.

CHAPTER 2

MATERIALS AND METHODS

2.1. CHEMICALS AND ENZYMES

Chemical	Source
General chemicals and organic compounds	BDH, Hopkins and Williams, Koch-Light Laboratories, May and Baker
Media	Difco, Oxoid
Biochemicals	Sigma
Antibiotics	Sigma
Agarose	Sigma,Bethesda Research Laboratories (BRL)
Biogel	Biorad
Restriction enzymes	BRL, Boehringer Mannheim
Sephadex G-50	Sigma
1,10 Phenanthroline	Sigma
Reverse Transcriptase	Life Sciences
Terminal Transferase	BRL
Oligo (dT) 18	New England Bio Labs
d(X)TPs	P.L.Biochemicals
Diethylpyrocarbonate	Sigma
Methyl Mercury	Ventron
beta-Mercaptoethanol	Sigma
Radiochemicals	New England Nuclear
Biofluor	New England Nuclear
Nitrocellulose	Schleicher and Schull

SDS	Serva
Oligo (dT) cellulose	BRL
Hybond Affinity Paper	Amersham
Pst I cut -'G'tailed pBR322	BRL
Dextran Sulphate	Sigma

2.2. ORGANISMS AND GROWTH CONDITIONS

Saccharomyces cerevisiae DBY 746, a,his3, ura3-52, leu2-3, leu2-112, trp1-289.

YPG-Medium 20g glucose, 20g bactopeptone, 10g yeast extract made up to 1l with distilled water and autoclaved at 120°C for 20 minutes.

YPG-Agar As YPG medium with 20g agar of Oxoid No1 agar per litre, added prior to autoclaving.

Yeast strains were grown in YPG medium at 30°C with vigorous shaking (250 rpm). YPG agar plates containing yeast cells were incubated at 30°C.

E.coli 1400: ED 8767, supE, supF, hsd5, met$^-$, recA,/L512, (Cami and Kouilsky,1978).

L-Broth: 10g tryptone, 5g yeast extract, 5g Nacl, 1g glucose; made upto 1l with distilled water, adjusted to pH7.0 with NaOH and autoclaved at 120°Cat for 15 minutes.

L-Agar: As L-broth without glucose and solidified by adding 10g/l oxoid No1 agar prior to autoclaving as before. Appropriate volumes of antibiotic stock solutions were mixed with L-broth or L-agar. Tetracycline was used at 10ug/ml, ampicillin at 50ug/ml, and chloramphenicol at 250ug/ml.

Bacterial cultures were grown in L-broth at 37°C with vigorous shaking (250 rpm). Plates containing 25ml L-agar with appropriate antibiotics were incubated at 37°C for 16-18 hours.

2.3. ISOLATION OF RNA

Glasswares used for RNA work were heat-baked at 200°C overnight to inactivate RNases. All but Tris containing solutions were treated with 0.1% (v/v) diethylpyrocarbonate and autoclaved at 120°C for 15 minutes. Tris containing solutions were prepared using baked glasswares and RNase-free distilled water. Gloves were worn wherever necessary.

2.3.1.Buffers:

RNA extraction buffer: 0.1M Tris, pH 7.5
0.1M LiCl
0.01M DTT (added fresh)

Phenol: contained hydroxyquinoline and saturated with 0.1M Tris, pH 7.5

Chloroform: chloroform:isoamyl alcohol at 24:1 (v/v)

Sodium acetate 3M, pH 7.5

Oligo(dT) cellulose Column buffer:

1: 0.5M NaCl, 10mM Tris, pH 7.5.
2: 0.1M NaCl, 10mM Tris, pH 7.5.
3: 10mM Tris, pH 7.5.

2.3.2. RNA From Fresh Cells:

RNA from yeast cells were isolated according to the modified procedures of Lindquist (1981). Yeast cells were harvested from 100 ml of an exponentially growing culture by centrifugation at 5000xg for 5 minutes. The pellet was resuspended in 5ml of RNA extraction buffer at 4°C. The suspension was added to a mixture of 14g of 400-500 micron diameter glass beads, 1.0ml 10% (w/v)SDS, 5ml of phenol, 5ml chloroform and vortexed continuously for 5 minutes. The mixture

was centrifuged at 15000Xg for 10 minutes.The aqueous phase was then removed and extracted twice with equal volume of phenol and chloroform. The aqueous phase was extracted twice with 2 volumes of ether. Finally, 2 volumes of ethanol and sodium acetate (pH 7.5) (to a final concentration of 0.25M) were added to the aqueous phase and the samples stored at -20°C.

2.3.3. RNA From Stored Cells:

Experimental cultures of yeast cells were stored by adding 2 volumes of ethanol (Losson and Lacroute,1979) and kept at room temperature for one week . If the cells were to be stored for more than a week, the cells were harvested from ethanol by centrifuging at 5000Xg for 5 minutes. The pellet was resuspended in 5 ml of RNA extraction buffer and cells were centrifuged at 5000Xg for 5 minutes. The supernatant was discarded and the pellet was stored at -70°C till use. When convenient, RNA was isolated from the stored cells as described above.

2.3.4. Isolation of Poly(A)+ mRNA:

Poly(A)+ mRNA was isolated from the total cellular RNA according to the modified procedures of Aviv and Leder (1972). RNA, stored in ethanol as a precipitate, was centrifuged at 15000Xg for 10 minutes, the pellet was washed at 4°C in 75% (v/v) ethanol and dried in a vacuum dessicator. The pellet was dissolved in 250ul of 10mM Tris pH 7.5, and the solution was made up to 500ul by adding NaCl to a final final concentration of 0.5M. 0.2g of oligo(dT) cellulose was swollen in 1ml of oligo(dT) column buffer 1 and the column poured in a pasteur

pipette plugged with siliconized glass wool. The column was equilibrated with buffer 1 . The sample was loaded and 15 fractions of 0.8ml with buffer 1, 5 fractions of 0.8 ml with buffer 2 and 5-8 fractions of 0.2 ml with buffer 3 were collected. 20ul was taken from each of the fractions to measure the Absorbance at 260nm using Ultrospec 4050 (LKB) spectrophotometer. The amount of RNA in each fraction was calculated assuming that 1 Absorbance unit at 260 nm corresponds to 42 ug RNA per ml. Each fraction was also spotted on to agar plates containing ethidium bromide (at a concentration of 10ug per ml) and viewed under ultraviolet light to check which fractions contained RNA. Fractions using buffer 3 which contained RNA were pooled and the RNA was precipitated by adding 2 volumes of ethanol and sodium acetate to a final concentration of 0.25M. The integrity of the mRNA was checked by electrophoresing through an 1% (w/v) agarose gel under nondenaturing conditions.

2.3.5.Use of Hybond-mAP:

SSPE (20X)	NaCl	174g
	$NaH_2PO_4.H_2O$	27.6g
	EDTA, Na salt	7.4g
	made up to 1 l (pH 7.4)	

Hybond-mAP (messenger Affinity Paper) (Amersham) is based on paper affinity chromatography. It has been diazotized and substituted covalently by polyuridylic acid. The 3' poly(A) sequences present in most mRNAs of eukaryotic origin allow hydrogen bonding to the poly(U) moiety on the Hybond-mAP. This

provides an alternative to the oligo(dT) cellulose column chromatography for the isolation of poly(A)+ mRNAs. Isolation of poly(A)+ mRNAs from total cellular RNA was carried out according to the manufacturer's protocol. The capacity to retain poly(A)+ mRNAs is approximately 30-40ug per cm^2 of Hybond-mAP.

1 cm^2 Hybond-mAP was cut, moistened in 2X SSPE, placed on a filter paper and dried. Multiple 2ul aliquots of total RNA (100ug/10ul) were added and allowed to dry between each application. After drying the filters were washed in 0.5M NaCl (5ml/cm^2) three times for 10 minutes each, once in 70% ethanol (v/v) for 2 minutes and dried. Then the filter was submerged in a minimum amount of distilled water and incubated for 5 minutes at 70°C. The Hybond-mAP was removed and the resulting solution contained poly(A)+mRNA, ready for further use.

2.4.ISOLATION OF DNA

2.4.1.Buffer:

TE: 10mM Tris, 1mM EDTA, pH 7.5.

Yeast DNA Extraction Buffers:

1: 1M Sorbitol
20mM EDTA
20mM KH_2PO4 pH 7.5

2: 0.15M NaCl
5mM KCl
1mM EDTA
0.2% Triton X-100 (v/v)
1M Sorbitol
20mM Na Hepes pH 7.5

3: 1mM EDTA
5mM Na Acetate
40mM Tris pH 7.5

STET buffer: 0.8% (w/v) Sucrose, 0.5% Triton X-100 (v/v), 50mM Tris, 1mM EDTA, pH 8.0.

Lytic Mix: Triton X-100 2% (v/v)
Tris 0.05M, pH 8.0
EDTA 0.06M, pH 8.0

2.4.2. Large Scale Preparations of Plasmid DNA:

Plasmid DNA from E.coli cells were isolated according to the modified procedures of Holmes and Quigley (1981). E.coli cells were harvested from 100ml stationary cultures by centrifugation at 5000Xg for 5 minutes at 4°C. The cells were resuspended in 25ml STET buffer and 1ml of a freshly made lysozyme solution (20ug/ml in STET buffer) was added and the mixture boiled for 5 minutes. The lysate was centrifuged at 15000Xg for 20 minutes at 4°C and the pellet was removed. 3 ml of

3M sodium acetate pH 7.5, and 12ml isopropanol were added to the supernatant which was then incubated at room temperature for about 15-20 minutes. The precipitate was collected by centrifugation at 15000Xg for 15 minutes at room temperature. The precipitate was washed with 70% (v/v) ethanol at -20°C and dried for 10 minutes in a vacuum dessicator. This DNA was then purified by equilibrium centrifugation on CsCl/ethidium bromide density gradients.

Ethanol precipitates of plasmid DNA were dissolved in 4.83 ml TE buffer (pH 7.5). For CsCl/Ethidium bromide gradient centrifugation, 5.0g CsCl and 0.33ml of ethidium bromide (3mg/ml) were mixed with 4.83ml of DNA solution and the density was adjusted to 1.58g/ml. The solutions were placed in Beckman quick-seal tubes using liquid paraffin to fill any remaining space in the tubes and then sealed. Samples were centrifuged in a VTi50 rotor in a Beckman L-8 ultra-centrifuge at 167,000Xg fOr 16 hours at 20°C. At the end of the centrifugation two bands were usually visible under UV light, the upper containing chromosomal DNA and relaxed forms of plasmid DNA, and the lower band containing covalently closed supercoiled plasmid DNA. The lower band was removed by inserting a syringe needle through the side of the tube and slowly drawing off the band. Ethidium bromide was removed from the DNA by repeated extraction (usually 6-8 times) with butanol (saturated with distilled water). The DNA solution was poured into a dialysis tube and dialysed for 16-18 hours in a beaker containing TE buffer to remove the CsCl. After dialysis the DNA was removed from the dialysis tube and 2 volumes of

ethanol and sodium acetate (to a final concentration of 0.25M) were added and stored at -20°C overnight. Finally, the DNA was collected by centrifugation at 15000Xg for 10 minutes at 4°C, the pellet was washed with 5 ml of 70%(v/v) ethanol, dried in a vacuum dessicator and dissolved in 500ul of TE buffer.

2.4.3 Small Scale Plasmid Preparation

Small scale plasmid preparations were performed for rapid preliminary characterizations of recombinant plasmids according to the modified procedures of Holmes and Quigley (1981). 5ml of L-broth containing tetracycline (10ug/ml) were inoculated with putative recombinant clones of E.coli cells and grown to stationary phase. 1.5ml of this stationary phase E.coli culture was centrifuged for 15 seconds in an eppendorf centrifuge and the pellet was resuspended in 350ul of STET buffer. 20ul of lysozyme (20mg/ml in STET buffer) was added to the cell suspension and mixed. The sample was boiled for 40-60 seconds and centrifuged for 10 minutes in an eppendorf centrifuge at 4°C. The pellet was discarded and 40ul of 3M sodium acetate and 400ul of isopropanol were added to the supernatant and mixed well. After 2 minutes the sample was again centrifuged for 5 minutes at room temperature. The pellet was washed twice with 70%(v/v) ethanol at -20°C and dried in a vacuum dessicator. The pellet was taken up in 50ul TE buffer and 10ul of this was used for restriction digests. The remainder was stored at -20°C for up to two weeks for future use.

2.4.4. Chromosomal DNA from Yeast

Yeast chromosomal DNA were isolated according to the modified procedures of Livingston and Hahne (1979). Yeast cells were grown to mid-exponential growth phase in YPG medium at 30°C with vigorous shaking (250rpm). Cells were harvested by centrifuging at 5000Xg for 5 minutes, resuspended in yeast DNA extraction buffer 1. and centrifuged again at 5000Xg for 10 minutes . The cell pellet was resuspended in yeast DNA extraction buffer 2 and 50ul of freshly prepard DTT (dissolved in buffer 2) was added to a final concentration of 2mM. Zymolyase (dissolved in buffer 2) was added to a final concentration of 10mg/ml and the mixture incubated at 37°C. Cell lysis was monitored by light microscopy. When the digestion was complete, SDS was added to a final concentration of 1%(v/v) and both NaCl and EDTA were added to a final concentration of 0.1M. The final volume of the solution was made to 5ml using buffer 3. Proteinase K was added to a final concentration of 250ug/ml and the solution incubated at 37°C for 90 minutes. One volume of phenol saturated with Tris (pH 7.5) and one volume of chloroform with isoamyl alcohol (24:1) were then added and vortexed for 5minutes. The mixture was centrifuged at 15000Xg for 10 minutes . The aqueous phase was removed and subjected to 2 phenol and chloroform extractions followed by 2 chloroform extractions. Preboiled RNase A was added (10ug/ml final concentration) and incubated at 50°C for 15 minutes. 2 volumes of ethanol were added and the stringy DNA precipitate was removed immediately with a pasteur pipette and transferred to an eppendorf tube. The excess liquid was discarded

and the pellet was dried in a vacuum dessicator and resuspended in 0.5ml TE buffer. Two volumes of ethanol and sodium acetate (to a final concentration of 0.25M) were added to the DNA solution and stored at -20°C.

2.5. DNA MANIPULATION

2.5.1. Restriction of Plasmid DNA:

Restriction of plasmid DNA was carried out in 20ul reaction volumes. The final reaction volumes contained

0.5 - 1.0ug DNA

0.1 volume of 10X appropriate buffer

1 -10 units of the enzyme

water to make up the volume to 20ul.

Complete digestion was achieved in 3 hours at 37°C. Digests were analysed by electrophoresis through agarose gels.

2.5.2. Ligation of Restriction Fragments:

Restriction enzymes used to digest the DNA were destroyed by heating to 65°C for 5 minutes. Ligation was carried out in ligation buffer, which contained 67mM Tris.HCl,pH 7.6, 10mM $MgCl_2$, 10mM DTT, 1mM EDTA, 0.4mM ATP. 0.1 unit of T4 DNA Ligase/ug DNA was added. The DNA concentration of ligation was 20ug/ml. Ligation mixtures were incubated at 14°C for 16-18hours. These were then diluted in distilled water for use in transformation.

2.5.3. Ligation in Low Melting Point (LMP) Agarose:

The restricted fragments were electrophroresed in 0.7% (w/v) LMP agarose using TBE buffer (2.7.1). Appropriate bands were cut and melted in 5 vol. of TE buffer at 65-70°C for 30 minutes. (This could be stored at -20°C till further use). 20ul of this was added to 10ul of the vector (approximately 20ug/ml). 23 ul of TE buffer was added along with 6 ul of 10X ligase buffer and 1unit of T4 DNA ligase to make up a final volume of 60ul. The mixture was incubated at 14°C for 18-24 hours and heated to 65-70°C for 5 minutes. 10ul portions were used for transformations.

2.6 . CONSTRUCTION OF cDNA LIBRARY

2.6.1.Buffers

cDNA buffer: 0.5M KCl, 0.05M $MgCl_2$, 0.25M Tris, pH 8.3

Homopolymer tailing buffer(10 X):

1.4M K-Cacodylate (chelexed),

5mM $CoCl_2$, pH 7.6.

TEN buffer: 0.1M NaCl, 1mM EDTA, 10 mM Tris, pH 7.5.

cDNA Reaction Mix: (for 10ug of Poly(A)+ mRNA)

cDNA buffer (pH 8.3)	30ul
dXTPs (10mM) 10ul of A,G,T each	30ul
Oligo (dT) 18mer (10mM)	7.5ul
RNasin (200 units)	10ul
^{32}P dCTP (60uCi)	6ul
RNase-free water	12.5ul
Total	96ul

2.6.2. Reverse Transcription:

Poly(A)+ RNA was reverse transcribed according to the modified procedures of Crabtree and Kant (1981). 5ug of Poly(A)+ mRNA, stored as precipitate in ethanol was centrifuged, washed twice with 70% (v/v) ethanol at -20^{o}C and dried in a vacuum dessicator. The RNA was resuspended in 13ul of RNase free water. Methyl mercury was added to this RNA solution to a final concentration of 4mM and incubated at room temperature for 10 minutes. The addition of 7ul beta-Mercaptoethanol was followed by the addition of 48ul of cDNA reaction mix and 20 units of reverse transcriptase giving a final volume of 71ul. The reaction mix was incubated for 60 minutes at 42^{o}C and stopped by adding RNase-free NaCl to a final concentration of 0.1M. The yield of the cDNA was estimated by TCA precipitations (See TCA precipitation). The reaction mixture which contained a complex population of mRNA:cDNA hybrids was passed through a Sephadex G-

50 column equilibrated with TEN buffer. 100ul fractions were collected and the radioactivity from 1ul portions of each fraction was measured by Cerenkov counting in a scintillation counter. The first peak, which eluted in the void volume of the column was pooled leaving the second peak which contained unincorporated nucleotides. Nucleic acids in the pooled fractions were precipitated with 2 volumes of ethanol in siliconized tubes.

2.6.3.Homopolymer tailing:

Homopolymer tails of 'C' residues were added to the 3' ends of mRNA:cDNA hybrids using terminal transferase according to the modified procedures of Deng and Wu (1983). Ethanol precipitates of the mRNA:cDNA hybrids were collected by centrifuging at 40,000 rpm for 1 to 2 hours in a precooled SW50.1 rotor at 4°C. The dried pellet was resuspended in 160ul of RNase-free water, and 100 units of RNAsin in 10ul was added along with 40uCi d(CTP). 40ul of homopolymer tailing buffer was added, and the volume was made up to to 360ul by adjusting with RNase-free water. This mix was incubated at 15°C for 15 minutes after which 160 units of Terminal Transferase in 40ul were added to bring the reaction volume finally to 400ul. Incubation was continued at 15°C for 30minutes. At this stage TCA precipitations were performed to estimate the number of 'C' residues added at the '3' ends of the mRNA:cDNA hybrids. At the

endof the reaction phenol, chloroform extraction was carried out twice, followed by two chloroform extractions. To the aqueous phase, 2 volumes of ethanol and sodium acetate (to a final concentration of 0.25M) were added and stored at -20°C.

2.6.4. Annealing With Vector:

Homopolymer 'C' tailed mRNA:cDNA hybrids, stored as ethanol precipitates were harvested by centrifugation at10000Xg for 10 minutes in an eppendorf centrifuge. The pellet was dried in vacuum dessicator and resuspended in water. Approximately 1ug of 'C' tailed mRNA:cDNA hybrids were added to PstI cut, 'G' tailed pBR322 (BRL) in 1:2 molar ratio and annealing was carried out in TEN buffer at the final DNA concentration of 20ug per ml at 65°C for 90 to 120 minutes.

2.6.5. Transformation of E.coli:

Transformation of E.coli cells were done according to the modified procedures of Mandel and Higa (1970). 10ml of a fresh stationary culture of E.coli 1400 was used to inoculate 1l of L-broth prewarmed to 37°C. This culture was grown with vigorous shaking (250 rpm) for about 90 minutes till it reached an A_{650} of 0.25. Cells were cooled on ice for 10 minutes and were harvested by centrifugation at 5000Xg for 5 minutes at 4°C. Cells were resuspended in 60ml of 100mM $CaCl_2$ (4°C) and kept at 4°C for 30 minutes. The cells were centrifuged at 3000Xg for 5 minutes and resuspended in 10ml of 100mM $CaCl_2$ at 4°C. After

incubation on ice overnight, the cells were either used for transformation immediately, or mixed gently with 0.25 volumes of glycerol and aliquoted into 50ul portions in eppendorf tubes for storage at -70°C. The cells were competent for at least 6 months after storage. Stored cells were thawed on ice for 20 minutes before use.

Approximately 40ng of DNA in 10ul of TE was mixed with 50ul of competent E.coli cells, incubated for 15 minutes at 0°C and then transferred to 37°C for 5 minutes. 1 ml L-broth was added and the incubation continued at 37°C for 30-40 minutes to allow the expression of the antibiotic resistance. Finally, 100-200ul of the transformation mixture was plated onto selective agar plates and incubated overnight at 37°C.

2.7.ELECTROPHORESIS OF NUCLEIC ACIDS

2.7.1. Buffer

MOPS buffer(10X): 0.2M Morpholinopropane sulphonic acid
0.05M Na Acetate,
0.01M EDTA, pH 7.0.
(Stored in dark bottle, without autoclaving)

TBE Buffer (10X): Tris base 108 g
Boric acid 55 g
EDTA(Na_2) 9.5 g per litre

Sample loading buffers:

Agarose gels:	Bromophenol Blue 25%
	Ficol (type 400) 15%
Alkaline agarose gel:	Glycerol 50%
	Bromocresol green 0.2%
Formaldehyde gel:	Glycerol 50%
	Bromophenol blue 0.4%
	Xylene cyanol 0.4%
	EDTA 1mM

MMF:	Formamide	500 ul
	Formaldehyde	162 ul (37%)
	MOPS	100 ul (10 X)
	H_2O	283 ul
	Total	1000 ul.

Alkaline Agarose gel

stock solution(10X):	0.3M NaOH
	20mM EDTA

2.7.2. Agarose Gels:

Sigma agarose (type II medium EEO) and TBE buffer were used to make 1% (w/v) horizontal gels for electrophoresis unlessotherwise stated. The agarose and TBE buffer (1X) were heated together at 100°C until the agarose was completely molten and cooled to 52°C. Ethidium bromide was then added (0.5ug/ml final concentration) and the gel poured, avoiding air bubbles. Once the gel was set, TBE buffer was poured to cover the gel. Loading buffer (2ul per 10ul of sample) was mixed with the DNA or RNA solutions and the samples loaded on to the gel. Gels were run at 5 V/cm at room temperature, unless otherwise stated and were viewed under UV light using a Chromato VUE transilluminator model TM 36 (Ultraviolet Products Inc.). The gels were photographed using a polaroid camera with a red filter (Wratten 23A)and polaroid film when necessary.

2.7.3. Alkaline Agarose Gels:

This type of gel was run to characterize the cDNA synthesised for preparation of cDNA libraries, according to the modified procedures of Favaloro et al. (1980). 1.5g of agarose (Type II Medium EEO) was melted in 90 ml water at 100°C, cooled to 52°C and 10ml of 10X alkaline agarose stock solution was added, whereupon the gel was poured immediately. The cDNA sample was dissolved in 2ul of the 10X alkaline agarose buffer, 4ul loading buffer added, and made up to 20ul with distilled water before loading on to the alkaline agarose gel. The gel was run at low voltage at 4°C with 1X stock solution as buffer.

2.7.4. Low Melting Point (LMP) Agarose Gels:

These gels were run when a particular DNA restriction fragment was to be isolated for use in cloning. LMP Agarose was melted in TBE buffer (1% w/v) at 65°C, and was cooled to 37°C. Ethidium bromide (0.5ug/ml final concentration) was added to this molten gel and the agarose was poured using a baby gel kit (BRL). 10-15ul of sample was loaded on to the gel using 2ul loading buffer and was run at 5 V/cm at room temperature. The gel was visualized under UV, as before. The gel band containing the required DNA was excised, melted at 65°C and diluted in five-fold TBE. This could be used for ligation without further purification.

DNA fragments were isolated from the agarose gels in the following way. The gel was placed on a long wave UV transilluminator and the relevant bands were excised with a scalpel. The gel slices were placed in a dialysis tube with a minimum amout of TBE buffer. The DNA was electroeluted at 50V for 2 hours after which the current was reversed for 2 minutes to remove DNA from the sides of dialysis tube. The DNA solution was removed from the dialysis tube and transferred to a 1.5ml eppendorf tube. 2 volumes of ethanol and sodium acetate was added (as before) to precipitate the DNA. The precipitate was collected by centrifugation in an eppendorf centrifuge. The pellet was washed twice with 70%(v/v) ehthanol at -20°C and dried in a vacuum dessicator. Finally the pellet was resuspended in TE. The DNA was ready for further manipulations.

2.7.5. Formaldehyde Gels:

These gels were for electrophoresis of RNA under denaturing conditions, and were run under RNase-free conditions according to the modified procedures of Goldberg (1980). 1.5g of RNase-free agarose was melted in 73ml of RNase-free water and cooled to 60°C. 10ml of 10X MOPS and 16.2 ml 37% (v/v) formaldehyde were added, mixed well, and poured. Combs (made RNase free) were inserted into the molten agarose and removed after the agarose was set. RNA solution was mixed with 8 volumes of MMF and the mixture was incubated for 15 minutes at 65°C to denature the RNA. The denatured RNA samples were mixed with formaldehyde gel loading buffer, loaded on to the gel and run at 100 V with 1X MOPS as running buffer with constant circulation of the buffer from anode to cathode to maintain a constant pH . After electrophoresis the gel was stained with acridine orange (30ug/ml in 1X MOPS) in the dark for 5 minutes, destained in distilled water for 10-15 minutes and viewed on a UV transilluminator.

2.7.6. Interpretation of the gel data:

The distance migrated by a given DNA molecule is dependent on its size and conformation. Generally, supercoils of a given length run farthest and open circles slowest whereas the linear moleculesrun between the two. The resolution of linear fragments of DNA are largely dependent upon the length, whereas the mobilities of RNA molecules under non-denaturing conditions dependent upon length and conformation. However, under denaturing

conditions the mobility of RNA depends upon the length. The mobility of known molecular weight size markers were plotted on a semilogrithmic scale from which the length of the unknown DNA or RNA molecules were estimated from their mobility.

2.8. FILTER HYBRIDIZATIONS

2.8.1.Solutions

5X SSCP:	NaCl	48.3 g	
	Na Citrate	22.1 g	
	Na_2HPO_4	9.5 g	
	NaH_2PO_4	4.8 g	per 1000ml

20X SSC:	NaCl	3.0M
	Na_3 Citrate	0.3M

100X Denhardt's solution:

BSA (Pentax fractionV)	2g
Ficoll (Mol.Wt.400 000)	2g
Polyvinyl pyrrolidone (Mol.Wt. 360 ,000)	2g in 100ml H_2O

ss DNA: Salmon sperm DNAs dissolved in sterile TE buffer at the concentration 5mg/ml, was needle sheared(21g) and stored in 2ml aliquotes at -20°C.

Prehybridization solution:

20 X SSC	10 ml
0.4M $NaPO_4$ (pH 7.0)	2 ml
50% Dextran Sulphate(w/v)	4 ml
100 X Denhardt's solution	2 ml
SS DNA	1 ml
0.25M EDTA, pH 8.0	1.6 ml
10% SDS (w/v)	2 ml
Formamide	14 ml
H_2O to	40 ml

2.8.2.Southern Blotting:

Southern blotting was done according to the procedures of Southern (1975). An agarose gel containing DNA, was gently shaken in 1.5M NaCl, 0.5M NaOH for 30 minutes to denature theDNA. The gel was washed briefly with 4 changes in distilled water and neutralised in 3M NaCl, 0.5M TrisHCl pH 7.5 for 30 minutes. The gel was placed on filter papers soaked in 20X SSC, in a tray containing 20XSSC. The nitrocellulose filter was placed on top of the gel followed by a stack of absorbent pads,on the top of which a weight of 1.5 Kg was placed. After blotting for about 16-18 hours the nitrocellulose filter was heat baked at 80°C for 3 hours.

2.8.3. Northern Blotting:

This was done according to the procedures of Thomas(1980). The formaldehyde gel containing RNA was stained with acridine orange to determine the distance migrated by size standards. The RNA gel was blotted and baked in the same way as for Southern blotting except for the omission of the incubation steps with alkali and Tris solutions.

2.8.4. Dot Blots For RNA:

RNA dot blots were made according to the modified procedures of Kafatos et al. (1979). An RNA precipitate was collected from the ethanol by centrifugation at 15000Xg for 10 minutes and the pellet was vacuum dried. The pellet was resuspended in 5ul of water. 8 volumes of MMF(2.7.1) was added. The mixture was incubated for 15 minutes at 65°C to denature the RNA. The denatured RNA was dotted onto a nitrocellulose filter in the following way.

Denatured RNA was diluted with 15X SSCP and the concentration was adjusted where necessary (usually 5ug of RNA per 100ul 15XSSCP). 100ul sample was dotted on to a nitrocellulose filter using a hybridot apparatus (BRL) using a gentle vacuum. After the samples were dotted on to the filter the top plate was disconnected and the filter was carefully removed while the vacuum was still on. The filter was dried and baked at 80°C for 3 hours.

2.8.5.In situ colony hybridization:

In situ colony hybridization was done according to procedures modified from Grunstein and Hogness (1975). E.coli cells from the cDNA library were plated on to tetracycline containing L-agar plates (120mm X 120mm)at the density of 10,000 colonies per plate and incubated overnight at 37°C. Colonies were transferred to duplicate nitrocellulose filters. The filters carrying the colonies were placed on L-agar plates containing tetracycline and the growth continued for 3-4 hours. Filters were then transferred to L-agar plates containing chloramphenicol and incubated at 37°C overnight for plasmid amplification. The filters were placed (colonies facing upwards) on top of 3-5ml puddles of lysozyme (10mg/ml) for 10 minutes. They were then transferred to 5ml puddles of 0.5M NaOH, 1.5M NaCl for 3 minutes. This step was repeated twice. Filters were then neutralised by transferring to 3-5ml puddles of 0.5M Tris HCl (pH 7.5), 3 M NaCl, twice for 3 minutes each. Then the filters were washed by immersing sequentially in 2XSSC containing 0.1% (w/v) SDS and then in 2XSSC for 5 minutes each. The filters were dried at room temperature for about 10 minutes and finally baked at 80°C for 3 hours.

2.8.6. Hybridizations:

Nitrocellulose filters upon which DNA or RNA was immobilised were sealed in plastic bags. Prehybridization solution (1ml per $5cm^2$ of nitrocellulose filter) was added and prehybridised for 3 hours at 48°C. Denatured, nick-translated DNA

probe was then added to the bag and hybridisation performed for 16-18 hours at 48°C. After hybridisation the filters were removed from the bag and washed as detailed below:

2 times in 2X SSCP + 0.5% SDS (w/v) for 15 minutes at 65°C

2 times in 1X SSCP + 0.5% SDS (w/v) for 15 minutes at 65°C

1 time in 0.1X SSCP + 0.5% SDS (w/v)for 15 minutes at 65°C

After the washes the excess liquid on the filters was allowed to drain, the filters were sealed in a bag and autoradiographed using X-Omat film with an intensifying screen at -70°C. After autoradiography the film was developed using Kodak developing fluids.

2.9. PREPARATION OF RADIOACTIVE PROBES

2.9.1.Buffers

Klenow buffer: 10mM $MgCl_2$, 10mM Tris,
0.066% Gelatin, pH 7.4. (w/v)

Nick Translation Mix:

Plasmid DNA (0.5ug-1ug in TE)	5ul
Klenow buffer	45ul
dXTPs (10mM;C,T,G) 2ul each	6ul
^{32}P-dATP (20uCi)	2ul
Beta-Mercaptoethanol 2.5%(v/v)	1ul
E.coli polymerase (5units)	1ul
Total volume	60ul

2.9.2. Nick-translation of plasmid DNA:

Plasmid DNAs were nick-translated according to the modified procedures of Rigby et al.(1977). To 60ul of the nick-translation mix 1ul of DNase (0.5ug/ml) was added and incubated at 14°C for 60 minutes. Care was taken that either the DNA or the DNase was added last. 5ul of 0.5M EDTA was added to stop the reaction along with dextran blue and phenol red. This was passed through a Sephadex G-50 column and the blue excluded fraction containing dextran blue and nick-translated DNA was collected, leaving behind the phenol red fraction which contained unincorporated nucleotides. A portion of this was subjected to scintillation counting to measure the the specific activity of the probe. These probes were boiled for 10 minutes just before use.

2.10. POLYSOME GRADIENTS

2.10.1. Buffers:

SED Buffer:	Sorbitol	1M
	EDTA	25mM, pH 8.0
	DTT	50mM (added fresh)
SEN Buffer:	Sorbitol	1.2M
	EDTA	10mM
	Na_3 Citrate	100mM pH 5.8

Lysis Buffer(10X):	Tris	100mM	pH 7.4
	NaCl	1.0M	
	$MgCl_2$	0.3M	

2.10.2. Preparation of polysome gradients:

Yeast polysome gradients were prepared according to the modidified procedures of Hutchison and Hartwell (1967). 10-50% (w/v) sucrose gradients were prepared under RNase-free conditions by mixing 10% and 50% sucrose solutions with the help of a gradient former. The mixture was dripped gently along the sides of SW28 Beckman centrifuge tubes. These gradients were stored at -70°C and thawed at room temperature before use in preparation of polysomes.

100ml YPG was inoculated with 4ul of a stationary culture of DBY 746 and grown at 30°C with vigorous shaking (250rpm) till the culture reached exponential growth phase (Absorbance of 0.45 at 650nm). The cells were then harvested by centrifugation at 5000Xg for 5 minutes, resuspended in 1/3 culture volume of SED and incubated at 30°C with gentle shaking. The cells were harvested by centrifugation at 5000Xg for 5 minutes, resuspended in 1/3 culture volume of SEN containing novozyme (Final concentration 20ug/ml) and incubated for 15 minutes at 30°C with gentle shaking. (Sphaeroplast formation was monitored using light microscopy before and after lysis with 1% (w/v) sarcosine). The sphaeroplasts were harvested by centrifugation at 2500Xg for 5

minutes at room temperature. The pellet was gently resuspended in 1/2 culture volume of YPG containing 1.2M sorbitol and incubated at 30°C for 90-120 minutes with gentle shaking to allow recovery of the sphaeroplasts after treatment with novozyme. The sphaeroplasts were harvested by centrifugation at 2500Xg for 5 minutes at room temperature, resuspended in 500ul of lysis buffer on ice, and this suspension was transferred to an RNase-free eppendorf tube. 10% (w/v) sodium deoxycholate was added and mixed rapidly by inversion followed quickly by the addition and mixing of 50ul of 10% (w/v) Brij 58. The lysate was centrifuged for 1 minute in an eppendorf microfuge at 10000 rpm and 500ul of the supernatant was layered on top of a 10-50% (w/w) sucrose gradient. The gradient was centrifuged in a SW28 rotor at 82,700Xg for 3-4 hours at 4°C in a L-8 Beckman analytical ultra-centrifuge and stopped without brake. The contents were fractionated through flow cell in a spectrophotometer (Ultrospec 4050, LKB) to monitor the A_{260} continuously. About 0.5 ml fractions were dripped directly into eppendorf tubes containing 900ul of RNase-free ethanol and 17ul of RNase-free 3M sodium acetate. The contents were mixed well and stored at -20°C. The RNA from these fractions were formaldehyde treated and used for dot blots.

2.11. IN VITRO TRANSLATION

1ug mRNA samples were translated in the presence of (^{35}S)-methionine(NEN) using a rabbit reticulocyte cell free

system (Amersham International plc). RNA translation was carried out in a mixture containing 80% (v/v) lysate with (^{35}S)-methionine to give a final concentration of 1uCi/ul. The samples were incubated at 30^{o}C for 90minutes. An equal volume of a RNase solution (RNase 2.5ug/ml, 40mM EDTA, 1% methionine) was then added and the samples were incubated for further 15minutes. The samples were assayed for the incorporation of (^{35}S)-methionine into protein products by TCA-precipitation and SDS-PAGE gels.

The (^{35}S)- labelled translational protein products were resolved on 9% (w/v) SDS-PAGE gels (Laemmli, 1970). 25ul SDS PAGE sample buffer (30% glycerol (v/v), 6% SDS (w/v), 15% (v/v) beta-mercaptoethanol, 150mM Tris.HCl, pH 6.7, 0.05% bromophenol blue) was added to the translation samples. The samples were boiled for 2 minutes and loaded on SDS-PAGE gels. The gels (15 X 20 X 0.2cm) were electrophoresed at 40mA for 4 hours then soaked for 1 hour in 10% (v/v) acetic acid,50% methanol (v/v) to fix the proteins in the gel. After fixing, gels were washed in 7% acetic acid then enhanced for autoradiography by soaking for 1hr in EN3HANCE (NEN). The gel was then washed in tap water for 1 hour,and dried (under vacuum) on to Whatman 3MM paper and exposed using Kodak X-Omat S film.

2.12. TCA PRECIPITATION

The incorporation of radioactive precursors into nucleic acids and proteins, either <u>in vivo</u> or <u>in vitro</u> can be measured by precipitating large molecules with trichloroacetic acid (TCA).

2.12.1. Filter precipitation

For measurement of incorporation into nucleic acids, 2.4cm discs of Whatman GF/C filters were spotted with 50ul of 10mM dATP or dCTP depending on which radiolabelled tracer was used in the assay. After drying, up to 5ul of sample was spotted onto each filter. The filters were washed in 5%(w/v) TCA at 4°C (50 ml per filter) for 30 minutes with stirring. This was repeated 3 times with fresh TCA. Then the filters were washed for 15 minutes in ethanol, ethanol: ether (1:1) and finally in ether with constant stirring. The filters were dried and the radioactivity in the TCA precipitates measured by scintillation counting.

The same steps were carried out for measuring incorporatiion of (^{35}S)-methionine into proteins except for the following modifications. Firstly, the filters were spotted with 10mM non-radioactive methionine. Secondly, after 3 TCA washes in 5% (w/v) TCA, the filters were placed in fresh 5% (w/v)TCA and heated for 100°C for 5 minutes to dissociate charged tRNAs. This was followed by a wash in 5% (w/v) TCA at 4°C for 15 minutes.

2.12.2. Whole cell TCA precipitation

An equal volume of 10% (w/v) TCA at 4°C was added to the cells at the end of the labelling period, and left for 30 minutes at 4°C. The precipitate was harvested by centrifugation (10000Xg)

for 5 minutes and the supernatant was discarded. The precipitate was resuspended in 5% (w/v) TCA and left for 20 minutes at 4°C. This was repeated 3 times. NaOH was added to a final concentration of 0.1M and incubated at 37°C for 15 minutes, for estimating the radioactivity incorporated into RNA. To this, TCA was added to a final concentration of 5% (w/v) and kept on ice for 20 minutes. The precipitate was recovered by centrifugation. 10 volumes of scintillant was added to one portion of the supernatant and the radioactivity was measured by scintillation counting.

The same steps were carried out for proteins except for the following. After 3 TCA washes the samples were boiled in TCA for 5 minutes to dissociate the charged tRNAs, and then incubated for 20 minutes at 4°C . The precipitate was harvested by centrifugation and the supernatant was discarded. The pellet was resuspended in small amount of water, 10 volumes of scintillant was added and the radioactivity was measured as before.

CHAPTER 3

THE CONSTRUCTION, ISOLATION AND CHARACTERIZATION OF YEAST cDNA CLONES

3.1.INTRODUCTION:

Ideally, a large number of individual mRNA species should be analysed *in vivo* to investigate factors which influence mRNA stability. Suitable probes are required to identify, quantify and characterize these mRNA species. The ideal way to create these probes is to make a cDNA library. A large number of procedures have been described for the construction of cDNA libraries. Therefore, a considerable amount of thought had to be given to chose a method which would yield a suitable cDNA library for this study and which would provide the most simple approach. Therefore, current methodologies are reviewed here.

cDNA molecules can be cloned into a number of vectors depending upon the experimental requirements. Convenient plasmid vectors such as pBR322 are commonly used since they contain single recognition sites for a number of different restriction enzymes, they are small in size and they carry selectable markers. Derivatives of phage lambda can also be used. These take advantage of the lambda packaging system which will enrich for clones containing large inserts if the appropriate lambda vector and experimental conditions are carefully chosen. A large number of lambda vectors have now been constructed (Murray *et al.*, 1977; Williams and Blattner, 1980). Collins and Brunning (1978) and Collins and Hohn (1979) have constructed novel hybrid vectors called cosmids, in which the lambda *cos* site is linked to a plasmid. These vectors combine the advantages of the lambda packaging system (and hence efficient size selection during

cloning) with all the technical advantages of plasmids. The filamentous coliphage, M13 can also be used as a cloning vector (Messing et al., 1977; Barnes, 1979). There seems to be no limitation on the length of the insert for M13 and the single-stranded-DNA can be readily isolated in a suitable form for rapid DNA sequencing.

cDNA is synthesised using RNA dependent DNA polymerase (reverse transcriptase) which was first found in RNA tumour viruses such as Avian Myeloblastosis Virus (Baltimore,1970; Temin and Mizutani,1970; Goodman and Spiegelman,1971). Reverse transcription is both primer and template dependent (Kacian et al., 1972; Verma et al.,1972) and produces a single-stranded DNA copy of the RNA (Verma et al.,1972). Since most eukaryotic mRNAs contain a poly(A) stretch at the 3'-end, oligo(dT) can be annealed to this poly(A) region to serve as a primer. The efficiency of this reaction, however, depends on the quality of the enzyme. The reverse transcriptase should be free of contaminating RNases since degraded RNAs do not serve as efficient templates (Baltimore,1970; Verma et al., 1972). The secondary structure of the mRNA affects the yield of reverse transcription, since the enzyme is unable to denature the secondary structure of the template RNA. Hence the mRNA template should be denatured by suitable agents such as methyl mercury to allow efficient reverse transcription.

The ratio of reverse transcriptase to mRNA template input influences the yield of full length cDNAs (Friedman and Rosbash, 1977). A 30-60 fold molar excess of the enzyme yields

maximum full length cDNAs and a pH of 8.3 is optimal for full length cDNA synthesis. A deviation of 0.5 pH units seems to result in a 5 fold decrease in the production of full length cDNAs (Maniatis et al., 1982). Ionic conditions affect the transcriptional efficiency of various templates, for example longer transcripts are obtained with potassium than sodium ions. The optimum potassium ion concentration is 140-150mM (Efstratiadis and Villa-Komoroff, 1979). Divalent cations are an absolute requirement for reverse transcriptase activity. The optimum Mg^{++} ion concentration is 6-10mM for efficient reverse transcription. High concentrations of the four nucleotides are also important for full length cDNA synthesis; 200-250 uM are generally used (Maniatis et al., 1982).

Synthesis of the second strand is often a poorly controlled step which it relies on the self priming ability of the 'hairpin loop' at the 3'-end of the cDNA strand. (Other methods do not rely on self-priming for second strand synthesis. Some of these are discussed later.) The second cDNA strand is synthesised using either E.coli polymerase I or reverse transcriptase following the removal of the RNA template either by mild alkali treatment or by digestion with RNases (Efstratiadis et al., 1976). The double-stranded DNA obtained in the reaction is treated with single-strand-specific S1 nuclease to remove the 'hairpin loop' structure at the 3'-end.

The double-stranded cDNA inserts are attached to the vector either by homopolymer tailing or with linkers. Homopolymer tailing takes advantage of the properties of calf thymus terminal

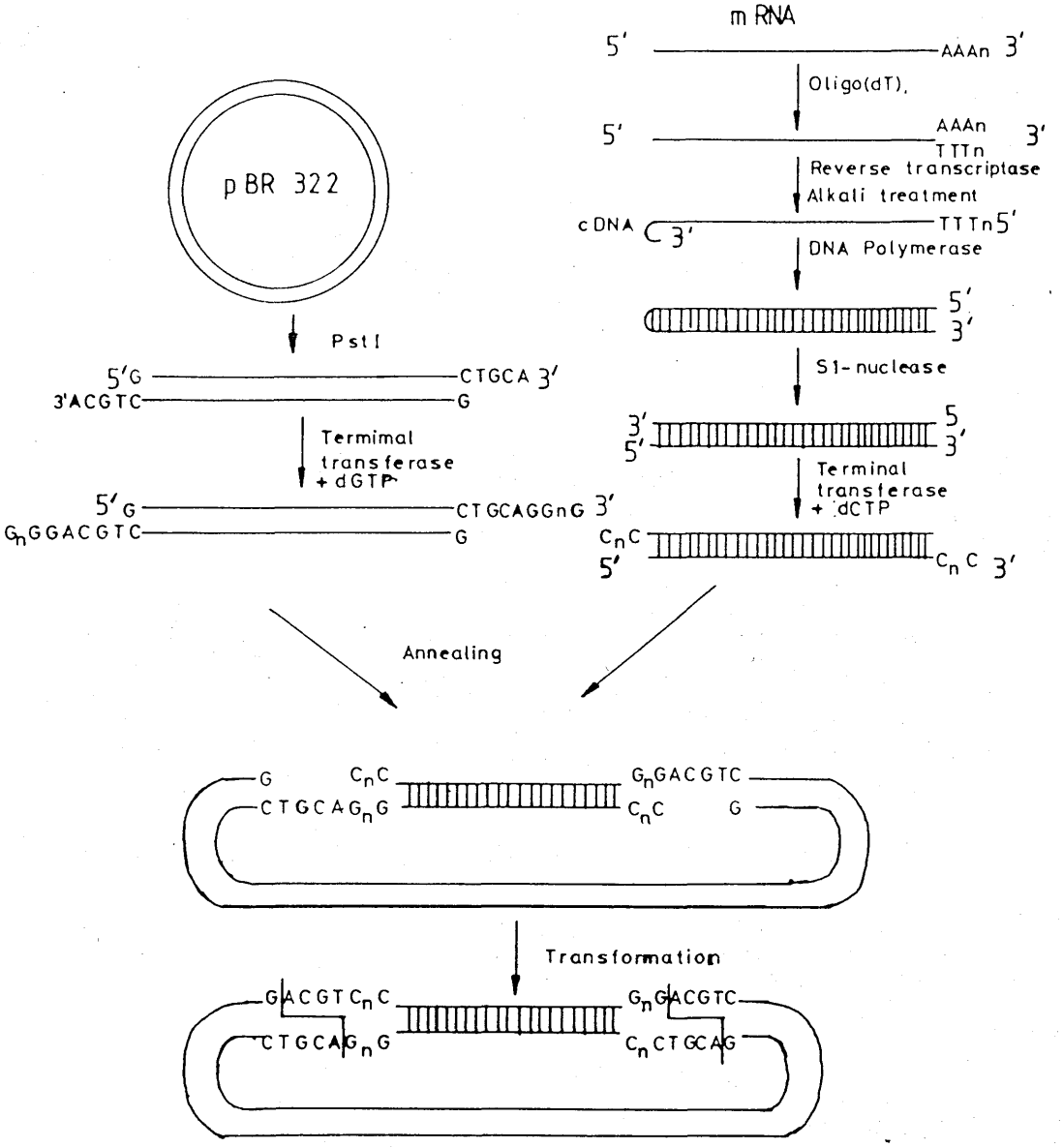


Fig. 3.1. Schematic diagram of double-stranded cDNA cloning in plasmid pBR322.

deoxynucleotidyl transferase which catalyses the addition of deoxynucleotides at the 3'-hydroxyl ends of single or double-stranded DNAs or DNA:RNA hybrids. In the presence of cobalt ions the enzyme efficiently catalyses the addition of the deoxynucleotides to either 'blunt' or 'protruding' 3'-ends (Roychoudhry et al., 1976; Deng and Wu, 1983). Some groups have used A:T tails, generally 50-150 residues in length (Jackson et al.,1972; Zain et al.,1979). However, in this case the isolation of the insert from the vector is generally tedious, involving the digestion of the recombinant molecules with S1 nuclease under moderately denaturing conditions at elevated temperatures (Hofstetter et al., 1976). Under appropriate conditions, the A:T tails denature and are cleaved by S1 nuclease, while the vector and cDNA denature less readily and are therefore, more resistant to S1 nuclease digestion. Alternatively the use of G:C tails has certain advantages. Unlike long A:T tails, short G:C tails (e.g. 20 residues) are sufficient for efficient annealing under standard conditions, and the inserts can be readily isolated from the vector (Villa-Komoroff et al., 1978; Rowekamp and Firtel, 1980). In general 'G' residues are added to the 3'-ends of a PstI cloning site of the vector and 'C' residues are added to the 3'-ends of the insert cDNA molecules. The annealing of such molecules results in the regeneration of the PstI sites at the boundaries of the insert in the recombinant plasmids. An advantage of homopolymer tailing for cDNA cloning is that it reduces the likelihood of self annealing of vector or cDNA

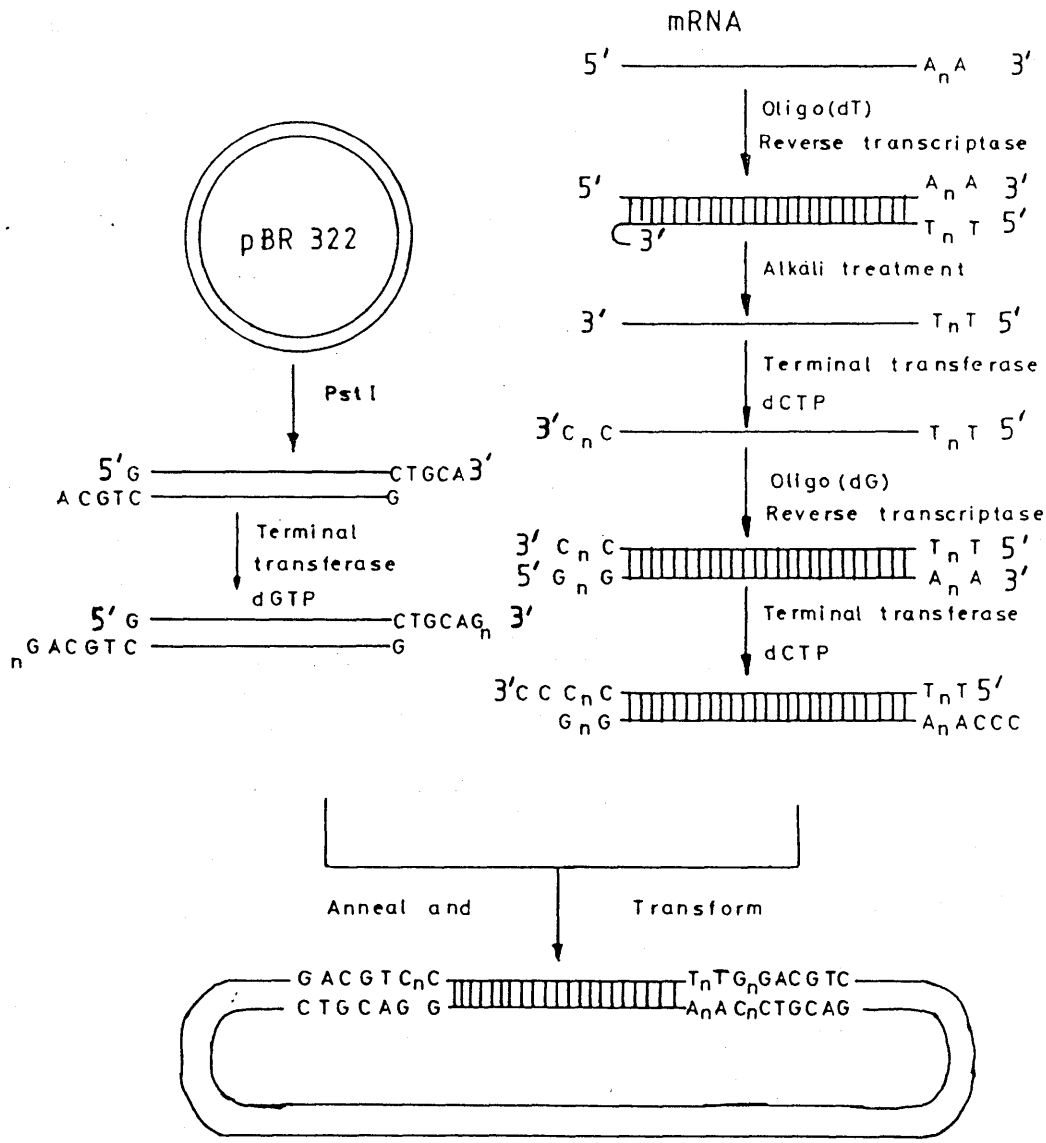
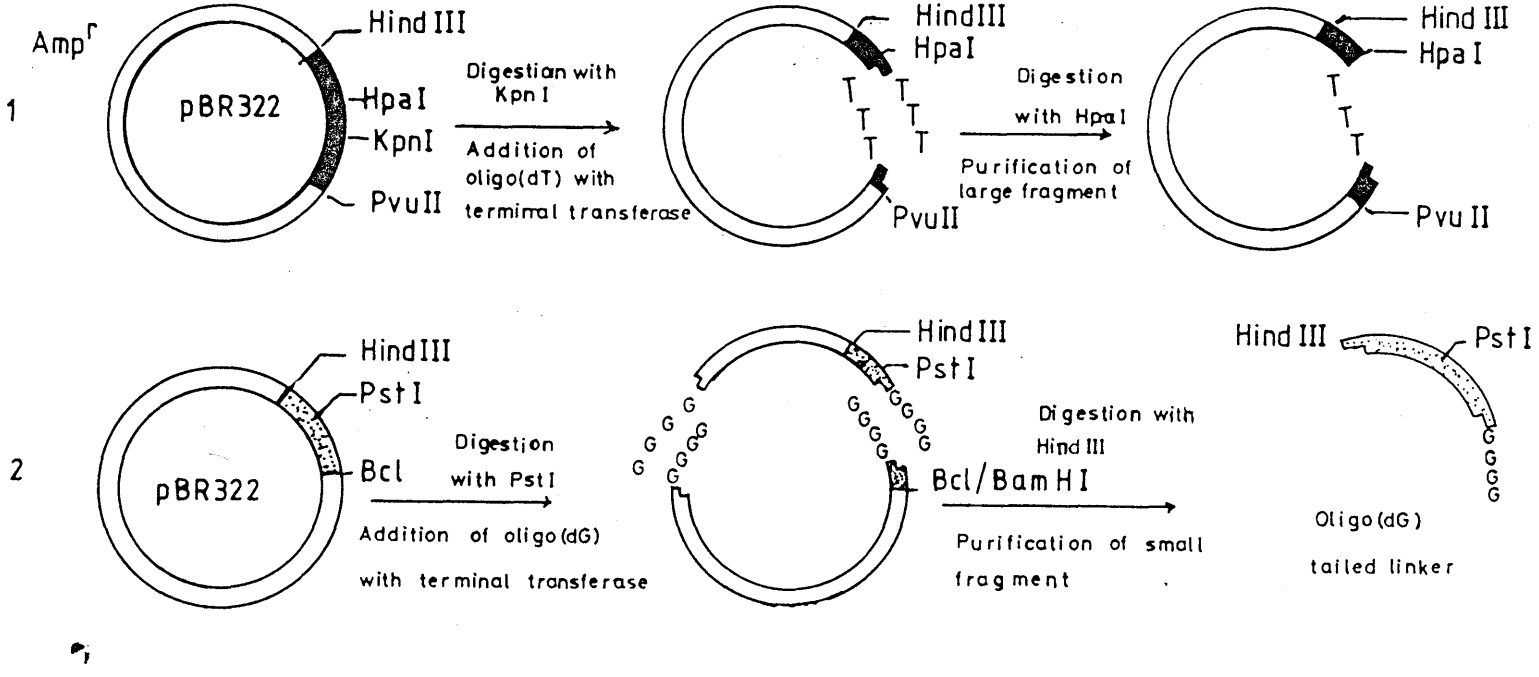


Fig.3.2. Diagramatic representation of the double-stranded cDNA cloning by oligo(dG) primed second strand synthesis (Land et al., 1981).

inserts. This method is also more convenient than the use of linkers because it involves less enzymic steps. The various steps involved in this protocol are shown in Fig. 3.1.

Land et al. (1981) developed a method whereby full length cDNA can be cloned with high efficiency, avoiding the use of S1 nuclease (Fig.3.2). This method is similar to that of Rougeon and Mach (1976). Poly(A)+ RNA is reverse transcribed and the mRNA is removed from the mRNA:cDNA hybrid by treating with alkali. The single-stranded cDNA is 'C'-tailed at the 3'-end using terminal transferase. Oligo$(dG)_{12-18}$ is hybridized to the 3'-poly(C) sequences of the cDNA and the second cDNA strand synthesised using reverse transcriptase. The double-stranded cDNA is again 'C'-tailed, annealed with PstI cut 'G'-tailed pBR322 and transformed into E.coli. By this method the authors were able to obtain full length cDNAs for chicken lysozyme mRNA. Though this method yields full length cDNAs,it involves a large number of enzymic steps.

Okayama and Berg (1982) have developed another method which circumvents the use of S1 nuclease and which yields full-length cDNA inserts. The steps involved in this protocol are shown in Fig.3.3. The first step involves the construction of a plasmid primer for cDNA synthesis. The appropriate plasmid is digested with a suitable restriction enzyme and 'T' residues are added to the 3'-ends using terminal transferase. This is followed by digestion with a second restriction enzyme to remove one of the 'T'-tails after which the large restriction fragment is purified. A 'G'-tailed linker DNA is also as prepared as shown


1
Amp^r
pBR322
Hind III
HpaI
KpnI
PvuII
Digestian with Kpn I
Addition of oligo(dT) with terminal transferase
HindIII
HpaI
PvuII
Digestion with HpaI
Purification of large fragment
Hind III
Hpa I
Pvu II
2
pBR322
HindIII
PstI
Bcl
Digestion with PstI
Addition of oligo(dG) with terminal transferase
HindIII
PstI
Bcl/Bam HI
Digestion with Hind III
Purification of small fragment
Hind III
Pst I
Oligo(dG) tailed linker

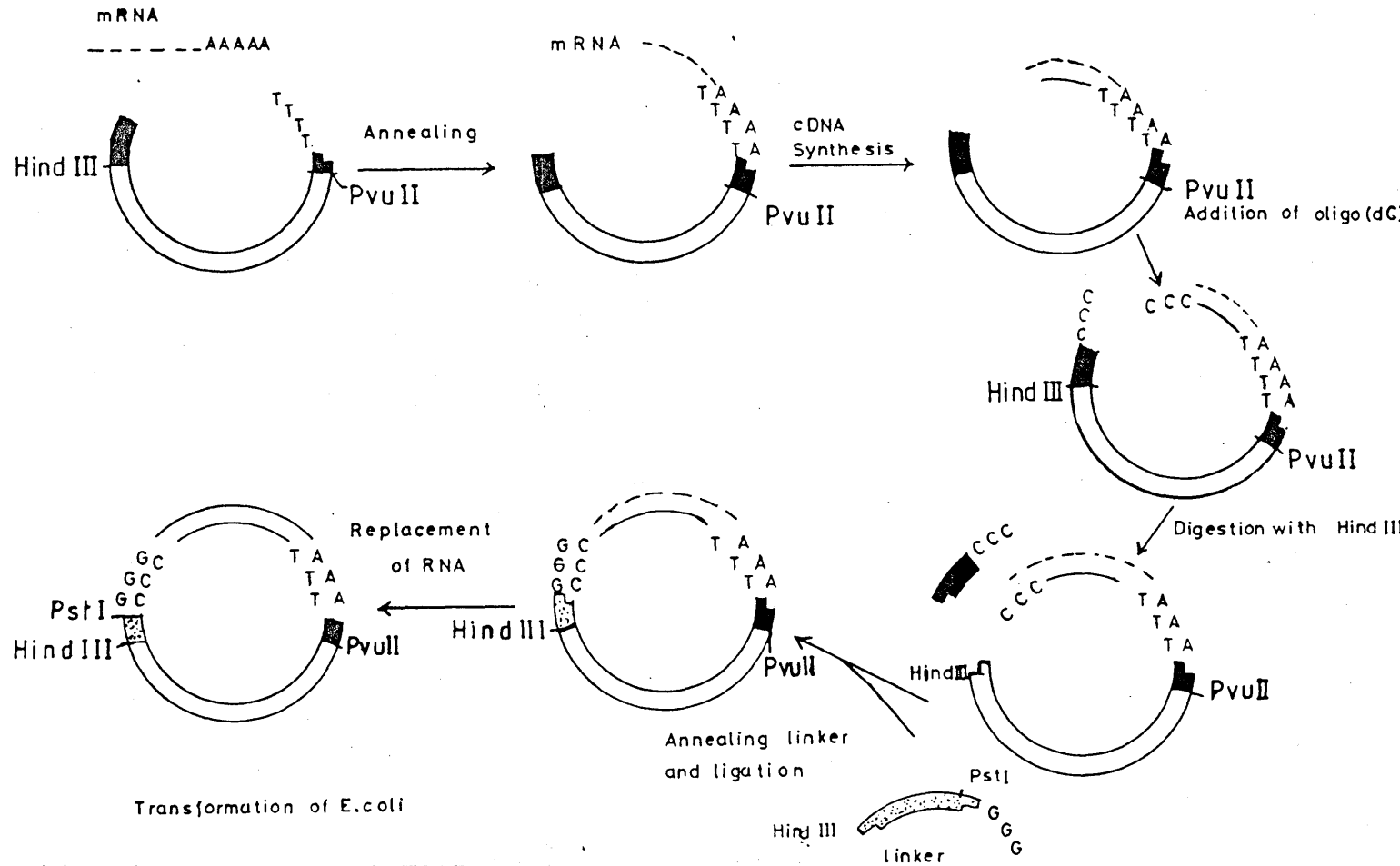


Fig.3.3. Diagram showing the various steps involved in the plasmid primed first and second strand cDNA synthesis (Okayama and Berg, 1982). Preparation of (1)-plasmid primer, and (2)-oligo(dG)-tailed linker. (3)-Construction of plasmid-cDNA recombinants. SV40 DNA is indicated by darkened or stippled segments.

in Fig.3.3. The 'T'-tailed plasmid primer is annealed with poly(A)+ mRNA and the first cDNA strand is synthesised using reverse transcriptase. The mRNA:cDNA hybrid plasmids are 'C'-tailed, the 'C'-tail removed from the non-cDNA end of the plasmid by restriction digestion and this construct is ligated to the 'G'-tailed linker DNA. In the next steps, the mRNA in the mRNA:cDNA hybrid plasmid is replaced by DNA using the combined activities of RNase H, E.coli polymerase I and DNA ligase. Though this method yields full or nearly full-length cDNA,it is fairly complex and requires specific plasmid vectors.

A simple method of cDNA cloning has been used by Wood and Lee (1976) and Zain et al. (1979). This method takes advantage of the observation that Col EI derived plasmids utilize RNA as a part of their replicative intermediate (Tomizawa et al., 1975; Oka and Inselberg, 1975). During replication, the mRNA sequences are replaced by DNA sequences by the host bacterial replication system. Using this principle, Wood and Lee (1976) successfully cloned rabbit globin mRNA:cDNA hybrids into E.coli C600. The protocol used by these authors is shown in Fig.3.4. The synthesis of the first cDNA strand was followed by homopolymer tailing. The 'A'-tailed mRNA:cDNA hybrid was annealed to the EcoRI cleaved Col EI DNA. After treating with DNA polymerase I and T4 DNA ligase, the mixture was transformed into E.coli. Zain et al. (1979) followed the same protocol except that pBR322 was used as the vector. This mRNA:cDNA hybrid cloning method is simple, but yields a low frequency of transformation.

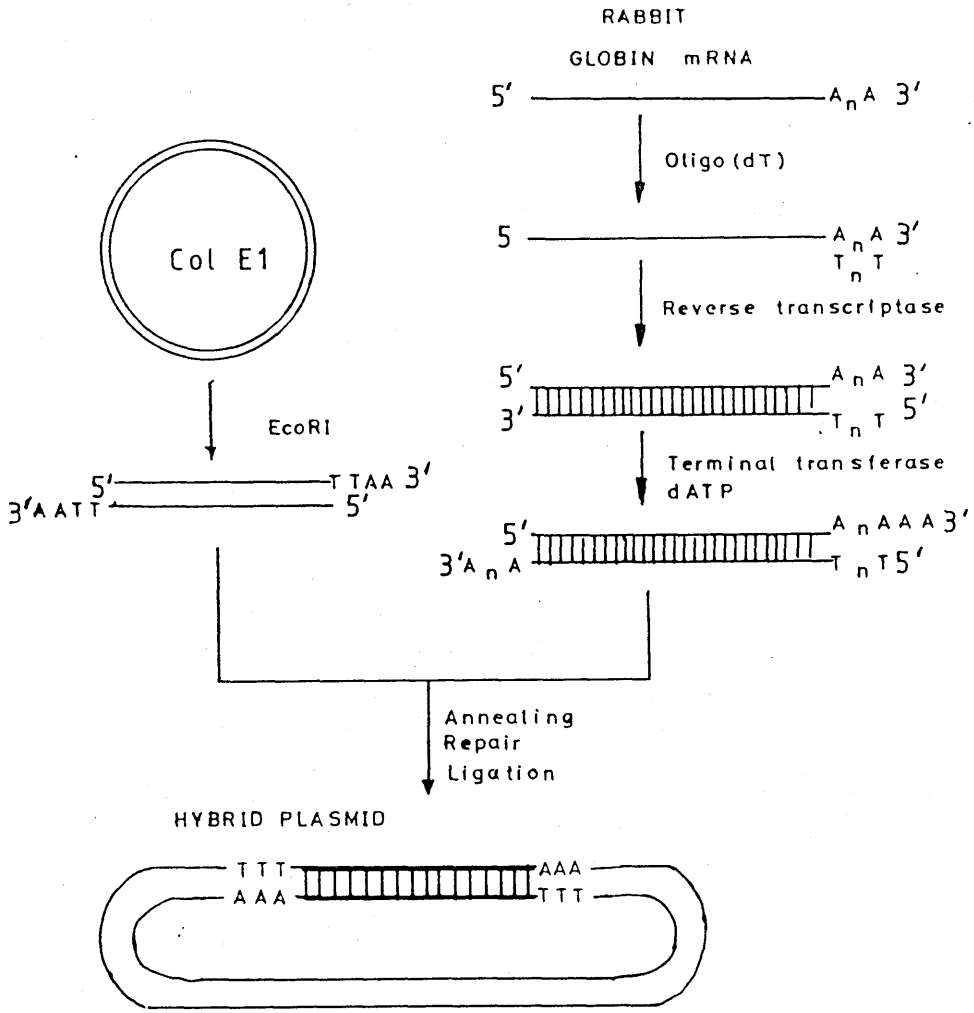


Fig.3.4. Diagram showing the various steps involved in rabbit globin mRNA:cDNA hybrid cloning in Col E1 plasmid.(Wood and Lee, 1976).

A number of other methods have been reported by various investigators (Spiegelman et al.,1971; Higuchi et al., 1976; Maniatis et al., 1976; Rabbitts, 1976; Rougeon and Mach 1976;Buell et al., 1978; Wickens et al., 1978; Gubler and Hoffman, 1983). Basically, they are modifications of those methods described above.

The primary reason for constructing a cDNA library was to create molecular hybridization probes for analysis of the behaviour of a variety of yeast mRNAs. Therefore, mRNA:cDNA hybrid cloning method modified from Wood and Lee (1976) was chosen because it is simple and rapid. Full length cDNAs were not an absolute requirement of this study. The vector of choice was pBR322 as it is small and hence the relative size of the cloned fragment compared to the vector is maximized. This is an advantage when using nick-translated recombinant plasmids as hybridization probes. Also, pBR322 is easy to purify in large quantities and it has a convenient PstI site for cDNA insertion. Finally it contains two selectable markers (ampicillin and tetracycline resistance) which allows recombinants to be selected by insertional inactivation. The protocol of for mRNA:cDNA hybrid cloning used in this study is shown in Fig.3.5.

One way to investigate factors which influence mRNA half-life is to compare the properties of mRNAs which have relatively long and short half-lives. Although such populations of yeast mRNAs were not available it was theoretically possible to isolate sequences from the cDNA library by differential screening. Differential screening has been used successfully to

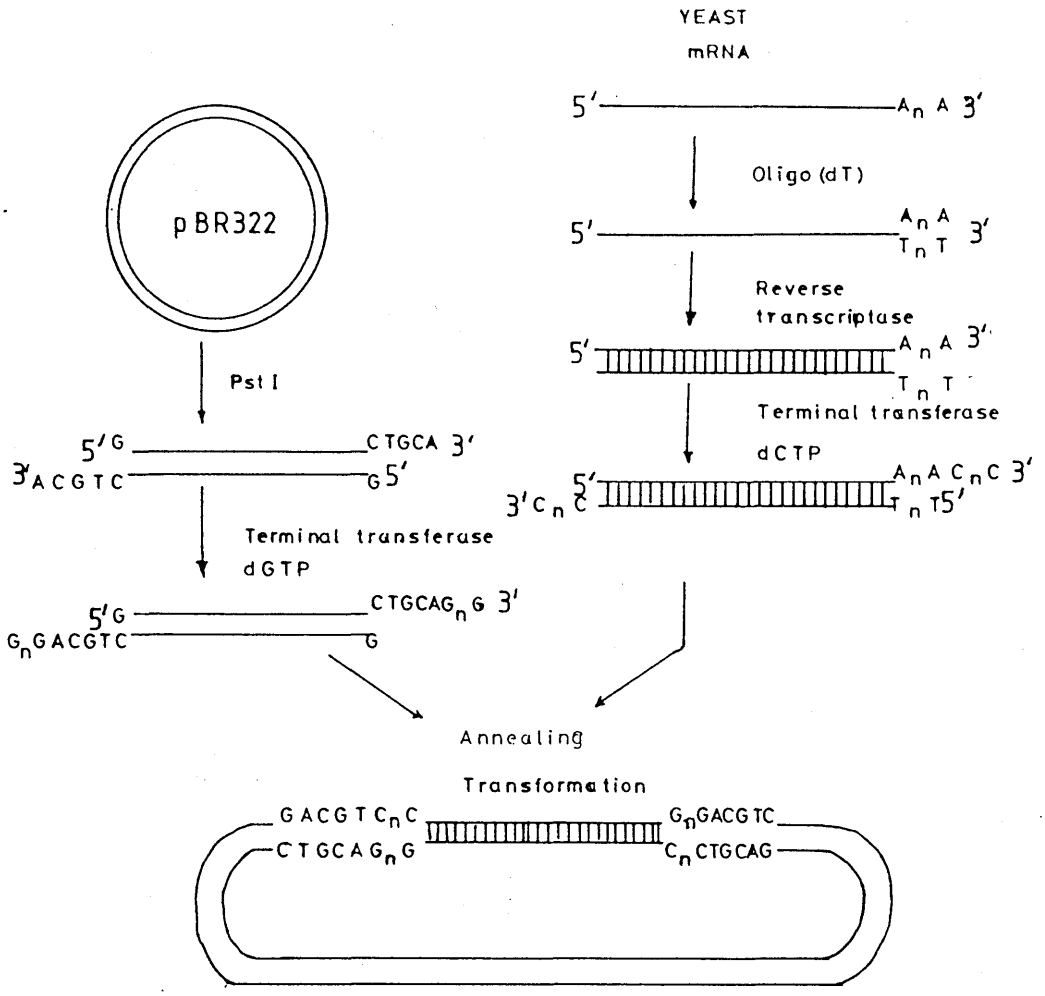


Fig.3.5. mRNA:cDNA hybrid cloning strategy used in this study. 'C'tailed mRNA:cDNA hybrid is annealed to the 3'end 'G'tailed (PstI site) pBR322 and transformed into E.coli1400. Tetracycline resistant, ampicillin sensitive colonies are selected as recombinants.

isolate sequences of interest from a wide variety of organisms. For example, St.John and Davies (1979) used differential screening procedures to isolate DNA sequences from galactose inducible genes in S.cerevisiae. Using a similar approach, developmentally regulated genes were isolated from the slime mould, Dictyostelium discoidium (Williams and Lloyd, 1979), from the sea urchin (Lasky et al., 1980) and from Aspergillus (Zimmermann et al., 1980).

It was envisaged that in this study cDNA clones which corresponded to mRNAs of long orshort half-lives, could be isolated from a yeast cDNA library by using differential screening with the following probes: firstly, a probe which contains a normal population of sequences derived from both long and short half-life mRNAs and secondly, a probe which is depleted in sequences from short half-life mRNAs. The first probe could be generated by cDNA synthesis using a normal population of poly(A)+ mRNA and it was theoretically possible to generate the second probe using poly(A)+ mRNA from heat-shocked yeast cells. Most eukaryotic cells respond to elevated temperatures by dramatically altering their patterns of protein synthesis. The synthesis of a small number of proteins is induced and they have been called 'heat shock' proteins, although it now appears that they are also induced in response to other forms of stress (Lindquist,1981). It has been shown that during heat shock in yeast, the transcription of the majority of genes is reduced while the transcription of heat shock mRNAs is induced (Lindquist,1981; Miller et al., 1982). Furthermore, most pre-

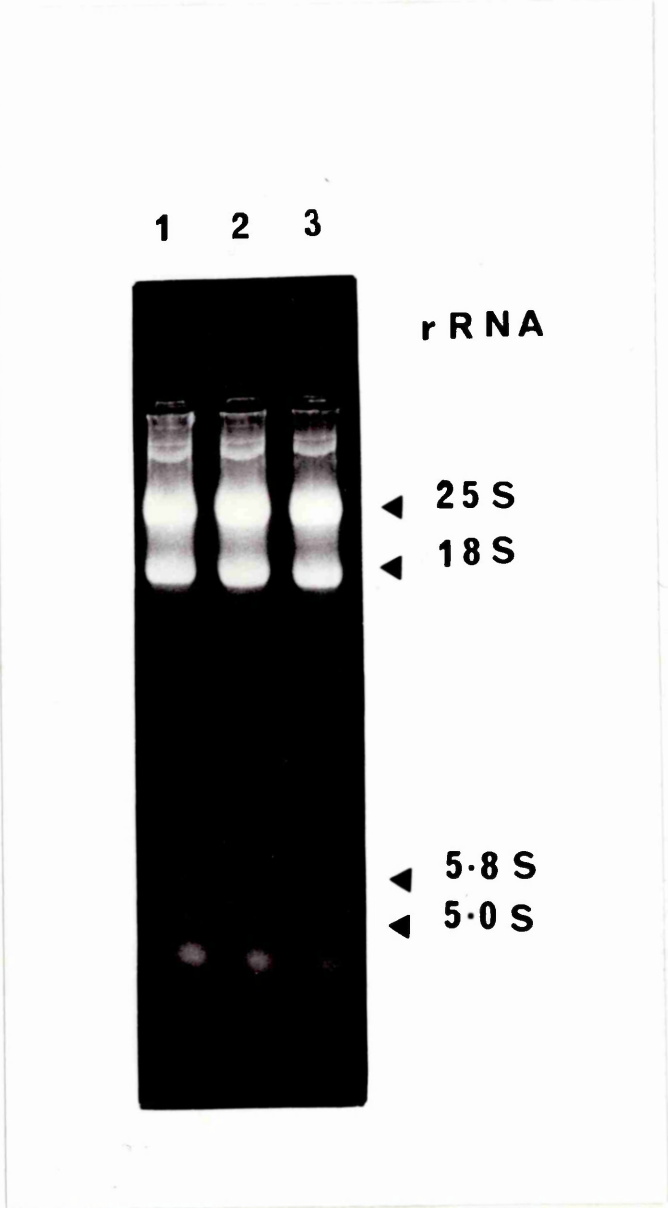


Fig.3.6. Electrophoresis of yeast RNA through an agarose gel under nondenaturing conditions. Total cellular RNA from S.cerevisiae was loaded in to each well of a 1% (w/v) agaorse gel and electrophoresed in TBE buffer. Each lane shows the presence of intact 25S, 18S,5.8S and 5S rRNAs.

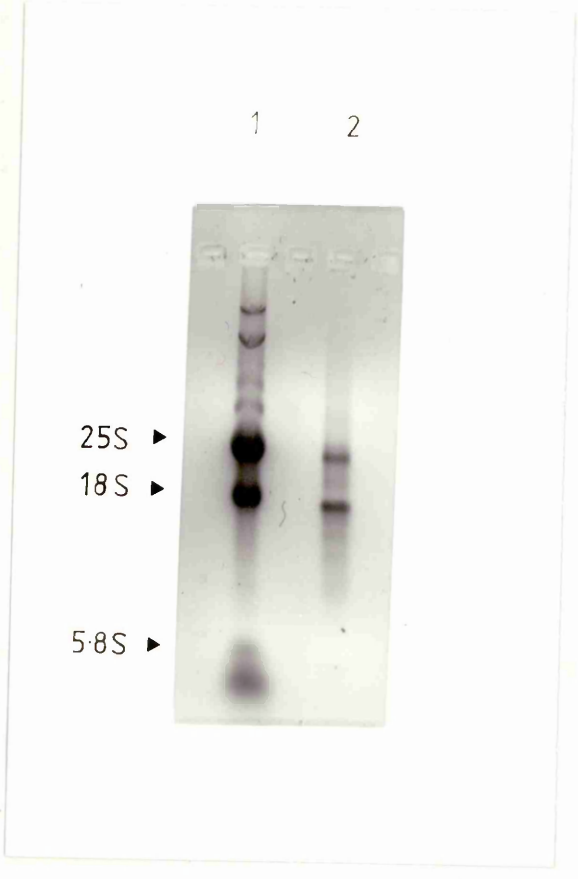


Fig.3.7. Electrophoresis of yeast poly(A)+ and poly(A)- RNA through an agarose gel (1%;w/v). Poly(A)+ and poly(A)- RNA were separated by binding to oligo(dT) cellulose column and eluting under different concentrations of NaCl as described in the text. Lane 1 shows the poly(A)- RNA. Lane 2 shows the poly(A)+ RNA still contaminated with 25S and 18S rRNAs.

existing yeast mRNAs appear to be degraded during heat shock, at what is assumed to be normal rates of turnover (Lindquist, 1981). Therefore, after heat shock a mRNA population would theoretically contain heat shock mRNAs, long half-life mRNAs and mRNAs which are continually transcribed during heat shock and would be depleted in short half-life mRNAs. Hence the poly(A)+ mRNA population from heat-shocked yeast cells can be used to synthesise a probe, depleted in short half-life sequences, for the differential screening strategy required in this study.

3.2. RESULTS

3.2.1. CONSTRUCTION OF YEAST cDNA LIBRARY

Total cellular RNA was isolated from exponentially growing yeast cells (DBY 746) as described in Chapter 2. When the integrity of the RNA preparation was tested by gel electrophoresis no smearing of rRNA species was observed. Therefore, it was assumed that the RNA was intact (Fig.3.6). Poly(A)+ mRNA was prepared from the total RNA using oligo(dT) cellulose affinity chromatography as described in Chapter 2. However, gel electrophoresis of the poly(A)+ mRNA showed that it was not free from contaminating rRNA (Fig.3.7.). The poly(A)+ RNA sample was about 5% of the total RNA, although this figure included rRNA contamination. This poly(A)+ RNA was used for cDNA synthesis, since the presence of the rRNA should not affect the yield of cDNA (Efstratiadis and Villa-Komoroff, 1979).

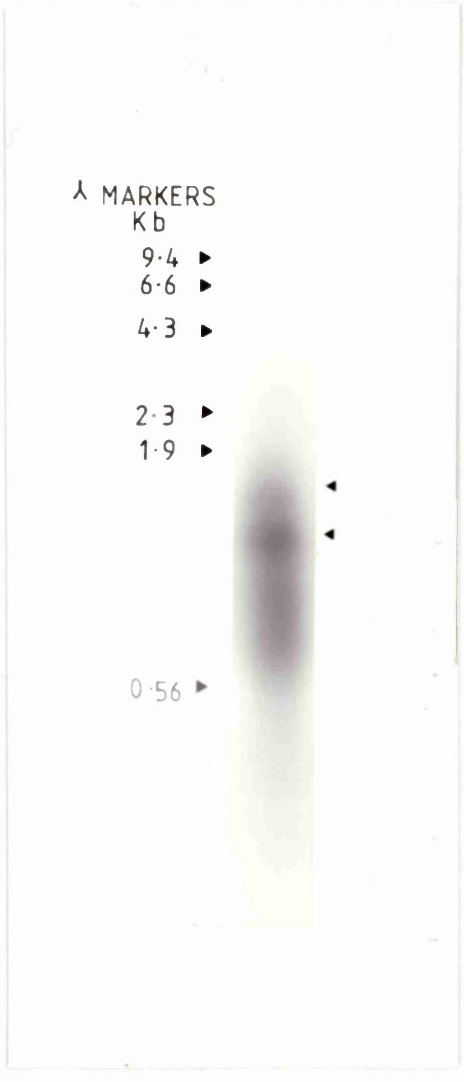


Fig.3.8. Autoradiograph showing the electrophoretic mobilities of the first cDNA strands. A portion of the cDNA reaction mixture was electrophoresed through an alkaline agarose gel (1.5% w/v) and the gel was dried under vacuum on a Whatman 3MM paper and exposed to X-omat film. Lambda HindIII digests are used as size markers. Presence of two bands are indicated on the right.

cDNA synthesis was carried out using 5ug of poly(A)+ RNA, following denaturation with methyl mercury as described in Chapter 2. RNAsin was added to the reaction mix to inhibit any contaminating RNase activity that might have been present in the reverse transcriptase. TCA precipitation of a portion of the reaction products indicated about 30% conversion and therefore, about 1.5ug of cDNA was synthesised. The length of the cDNA was estimated by alkaline agarose gel electrophoresis. By comparison with appropriate size standards, the length of the cDNA was estimated to range from 600-1900 bases (Fig.3.8). Two bands were visible, above the smear of cDNA, on the alkaline agarose gel (Fig.3.8). These two bands are unlikely to be due to rRNA contamination, since their lengths do not correspond to those of 18S and 25S rRNA. Also the reverse transcription was primed using oligo(dT) which does not have homology with these rRNAs (Rubtsov et al., 1980; Georgiev et al., 1981). It is likely that these bands arose via reverse transcription of abundant mRNAs (for example the PGK mRNA is about 1-5% of the total mRNA population; Tuite et al., 1982).

A series of control experiments were carried out to standardize the conditions for homopolymer tailing. The average length of the homopolymer tails was estimated using radiolabelled precursors followed by TCA precipitation. Using the conditions described in Chapter 2, approximately 15 'C' residues were added per 3'-end of the mRNA:cDNA hybrid (assuming an equal rate of addition to RNA and DNA ends). 'G'-tailed,PstI-cut pBR322 and 'C'-tailed mRNA:cDNA hybrids were mixed in the ratio of 2:1 at a

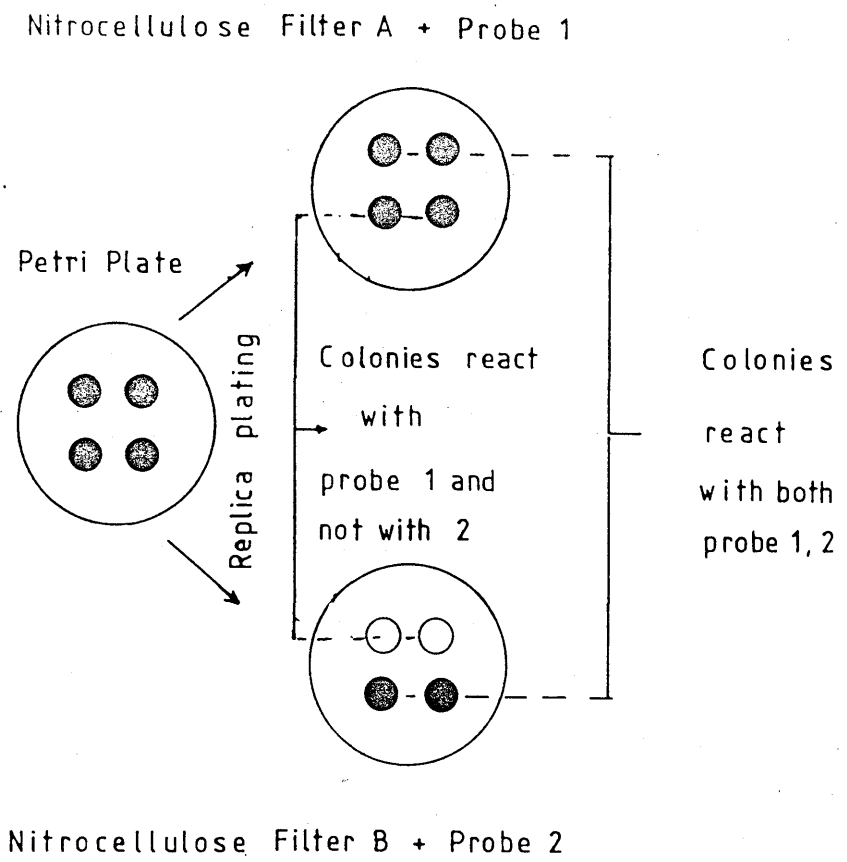


Fig.3.9. Schematic diagram of the principle of differential screening. ● on the petri plate represents bacterial colonies, whereas ● on the filter A and B represents colony that reacts with the hybridization probe. ○ represents colonies that does not react with the hybridization probe. Colonies that react with both the probes to the same degree contain sequences that correspond to long half-life mRNAs. Colonies that react strongly with probe 1 but not with probe 2 contain sequences that correspond to short half-life mRNAs.

final concentration of 20ug DNA per ml. Annealing was performed at 42°C for 90-120 minutes after heating to 65°C for 5 minutes. The annealing mix was then used to transform competent E.coli 1400 cells as described in Chapter 2 . A transformation frequency of 10^5 tetracycline resistant colonies per microgram of mRNA:cDNA hybrid was obtained. Replica plating of 100 tetracycline resistant transformants onto ampicillin plates showed that the level of background transformation due to non-recombinant pBR322 was less than 5%. The cDNA library of 150,000 colonies was collected and stored at -70°C in 2ml aliquots at a cell density of approximately 10^{10} cells per ml in L-broth containing tetracycline (10ug per ml) and glycerol (25%; v/v).

The range of lengths of cDNA insert obtained in the library were sufficient for use as hybridization probes.The length of the cDNA inserts ranged from 420-1200 base pairs (Table 3.1). A drawback in this method was that a proportion of the clones did not contain two regenerated PstI sites bordering the cDNA insert (about 13%). However, the inserts from all the clones used in this study were isolatable by PstI digestion (Fig.3.15).

3.2.2. DIFFERENTIAL SCREENING

The strategy for the differential screening of the yeast cDNA library is shown in Fig.3.9. Clones which contain sequences which hybridize to both probes probably represent long half-life mRNAs and clones which contain sequences which hybridize to first probe but not with the second probe may correspond to short half-life mRNAs. Yeast cells were heat-shocked according to the

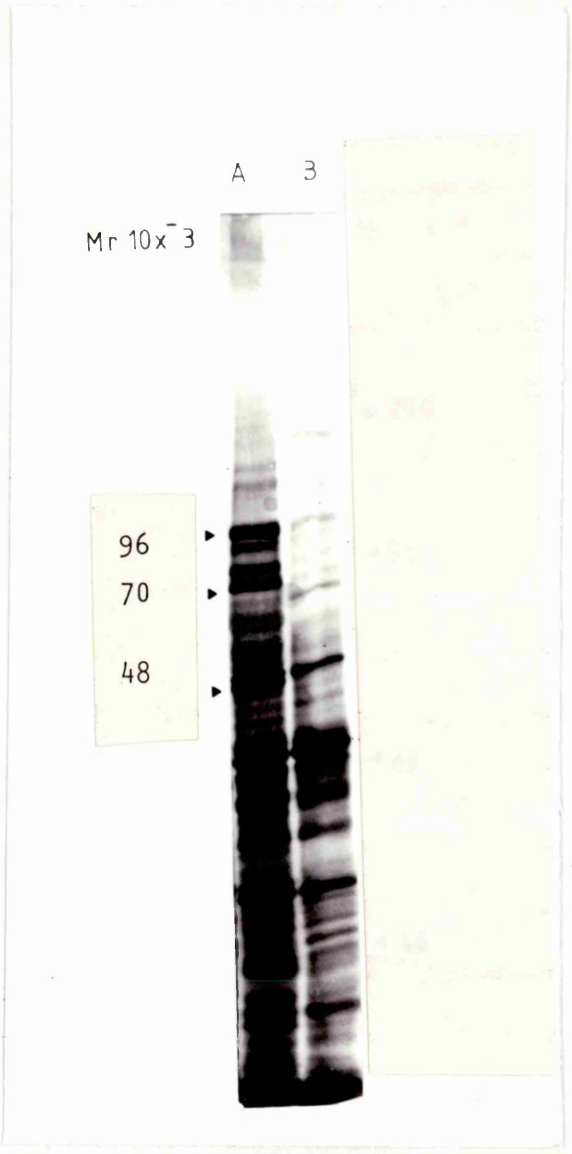


Fig.3.10. Autoradiography of the (^{35}S) methionine labelled <u>in vitro</u> translation products separated by 10% (w/v) SDS-Polyacrylamide gel electrophoresis. 1ug of poly(A)+ RNAs from heat-shocked and normal yeast cells were <u>in vitro</u> translated in rabbit reticulocyte lysate translation system as described in Chapter 2. An equal volume of each reaction mix was boiled for 5 minutes and loaded on to the gel and electrophoresed. Lane A shows the proteins synthesised using heat-shock poly(A)+ RNAs and lane B shows the proteins synthesised using poly(A)+ RNAs from normal yeast cells. Major new protein bands and their approximate molecular weights (96,000, 70,000 and 48,000 Daltons) are indicated on the left.

modified procedures of Ingolia et al. (1982). In brief, yeast cells were grown in YPG medium at 23°C with vigorous shaking (250 rpm) to mid-exponential growth phase (A_{650}= 0.45), whereupon the flask containing the yeast culture was immersed in a water bath at 50°C. Within 2 minutes the culture temperature rose to 36°C and immediately the culture was transferred to another water bath at 36°C. The culture was incubated at 36°C for 20 minutes with shaking, after which the cells were harvested and RNA and poly(A)+ mRNA were isolated as described in Chapter 2. Poly(A)+ mRNA from heat-shocked and normal yeast cells were translated in vitro using the rabbit reticulocyte lysate system as described in Chapter 2 to check the heat shock response. The in vitro translation products were electrophoresed through an SDS-Polyacrylamide gel, processed and autoradiographed as described in Chapter 2. Fig.3.10. shows the in vitro translation patterns obtained for normal and heat-shocked yeast poly(A)+ mRNA. The lane containing heat-shocked translation products shows the appearance of new polypeptides as well as the disappearance of some others relative to the control lane. The sizes of these new polypeptides (100k daltons, 90k daltons and 79k daltons) correlated well with the molecular weights of the major yeast heat shock proteins (Finkelstein et al., 1982). These results suggest that the yeast cells had responded to heat shock. Therefore, these preparations of poly(A)+ mRNA from normal and heat-shocked cells were used as templates to make the cDNA probes for differential screening.

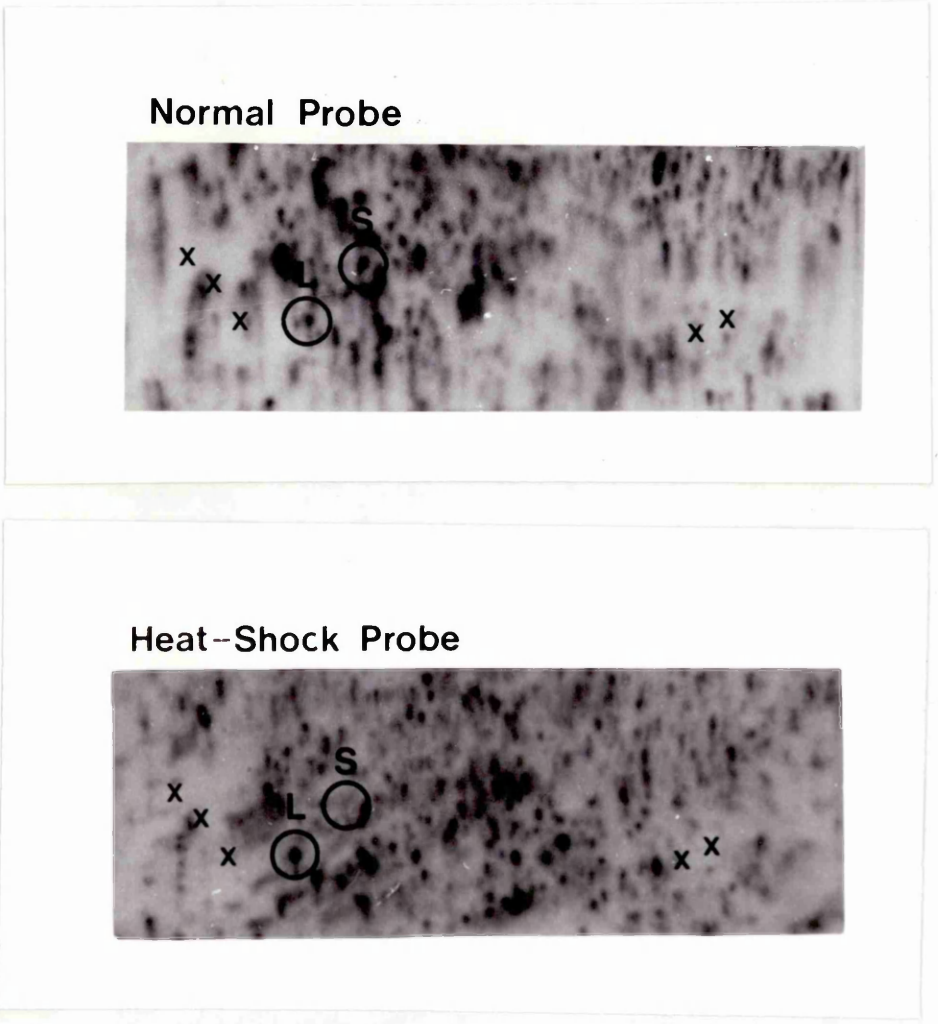


Fig.3.11. Isolation of cDNA clones by differential screening. Cells from yeast cDNA library were transferred to duplicate nitrocellulose filters and probed separately with probes 1 (Heat-shock) and 2 (Normal). L -colony that reacts with the probes 1 and 2 (corresponds to putative long half-life mRNA). S- colony that reacts with probe 2 (putative short half-life mRNA). (X)-marks for aligning the replica filters.

Approximately 40,000 colonies from the yeast cDNA library were transferred in duplicate to nitrocellulose filters and the filters were prepared for colony hybridization as described in Chapter 2. Duplicate filters were probed with heat-shock and normal cDNA probes (Fig.3.11). As described above, colonies which contained sequences that hybridized with both probes probably corresponded to long half-life mRNAs whereas those that hybridized only with the 'normal' cDNA probably corresponded to short-lived mRNAs. About 32 colonies were picked up and purified further. After two further rounds of screening only three clones reacted consistently in a manner which suggested they contained short half-life sequences (cDNAs 9,13 and 19) and only one appeared to be derived from a long half-life mRNA (cDNA11). This disappointing result may have been due to the possibility that the assumptions regarding mRNA turnover during heat shock in yeast (and hence the differential screening) were invalid. This was confirmed when the half-lives of mRNAs 9,11,13 and 19 were measured (Chapter 5). For example, the half-life of mRNA 11 (which was expected to be relatively stable because of the behaviour of cDNA 11 in the differential screening) lay within the experimental error for the half-life of mRNA 19 (a 'short half-life mRNA). The half-lives were 18.0 $\pm$ 2.2 and 15.3 $\pm$ 2.7 minutes respectively (see Chapter 5). As an alternative strategy to the differential screening, additional cDNA clones were randomly chosen from the cDNA library. The only prerequisite

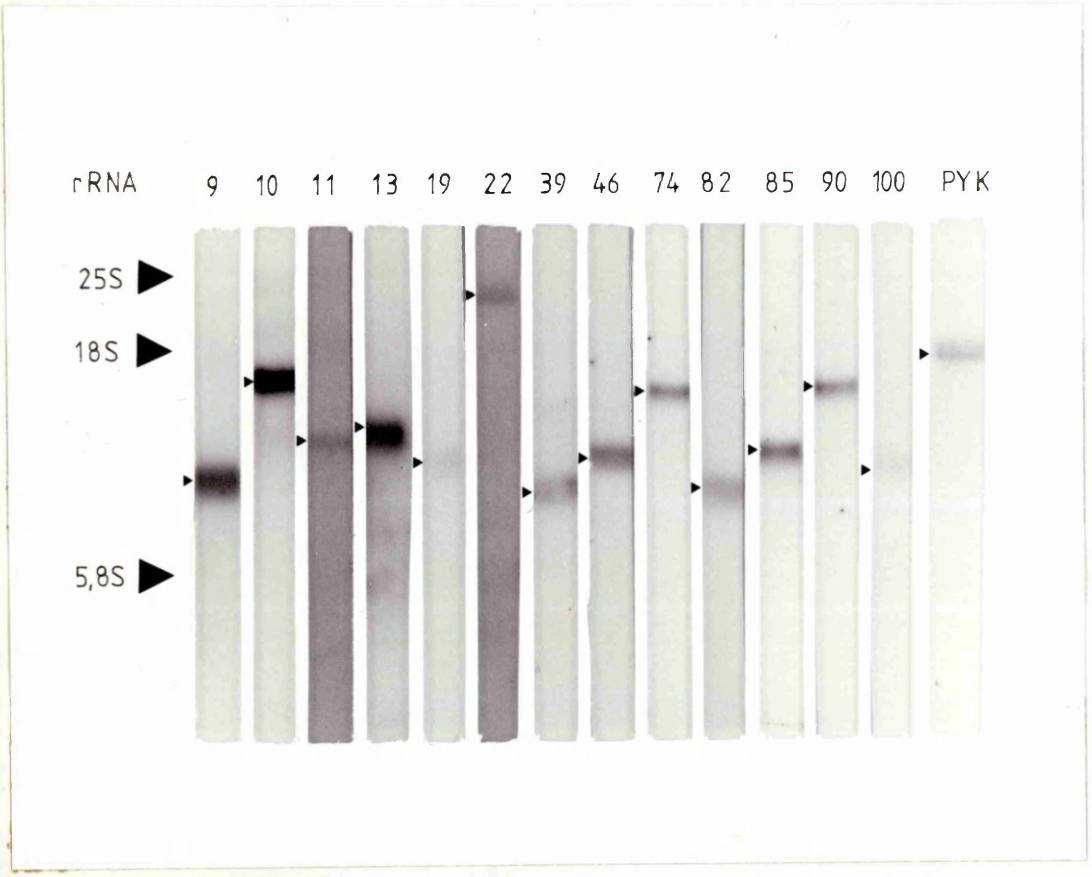


Fig.3.12. Northern blot analysis of the 14 mRNA species used in this study. The electrophoresis, blotting and probing of individual lanes containing approximately 5ug of total RNA are described in Chapter 2. The number above each lane corresponds to the number of the cDNA clone whose plasmid DNA was used as hybridization probe, apart from PYK, which relates to the pyruvate kinase genomic clone. Relative positions of 25S, 18S and 5.8S rRNA are indicated.

cDNA CLONE	mRNA LENGTH (BASES)	cDNA INSERT LENGTH (BASE PAIRS)
9	440	650
10	1,250	1,200
11	700	420
13	740	600
19	550	480
22	3,100	740
39	380	560
46	550	720
74	1,050	1,000
82	370	560
85	550	640
90	1,100	1,000
100	500	560

Table 3.1. The approximate length of mRNAs and corresponding PstI fragments of the cDNA inserts used in this study.

was that they contained PstI sites at the boundaries of the cDNA insert. This was to facilitate purification of insert sequences. These were cDNAs 10,22,39,46,74,82,85,90 and 100.

3.2.3. CHARACTERIZATION OF THE cDNA CLONES

It is possible that some of the cDNAs chosen by random picking and differential screening were derived from common mRNA sequences. Obviously, there is no point in repeatedly analysing the same mRNA using different cDNAs and hence these cDNAs should be excluded from the analysis. Also, cDNAs which contain highly repetitive sequences should be excluded since they may react with multiple mRNA species and thus would not be suitable for dot blotting experiments. Therefore, the cDNAs were characterized using northern and Southern blotting. Total yeast RNA was electrophoresed on denaturing gels and transferred to nitrocellulose filters as described in Chapter 2. After transfer, individual lanes from the northern blot were probed separately with the nick-translated cDNAs. The filters were washed under high stringency and autoradiographed as described in Chapter 2 (Fig 3.12.). All cDNAs gave a single band on the northern blots. Table 3.1 shows the length of each mRNA as estimated by comparison with the mobility of 25S, 18S and 5.8S yeast ribosomal RNAs. The lengths of the various mRNAs ranged from 370 to3100 bases, the majority being less than 1000 bases in length.

Yeast chromosomal DNA digested with EcoRI, was subjected to Southern blotting as described in Chapter 2. After the transfer, each lane was probed separately with one of the nick-

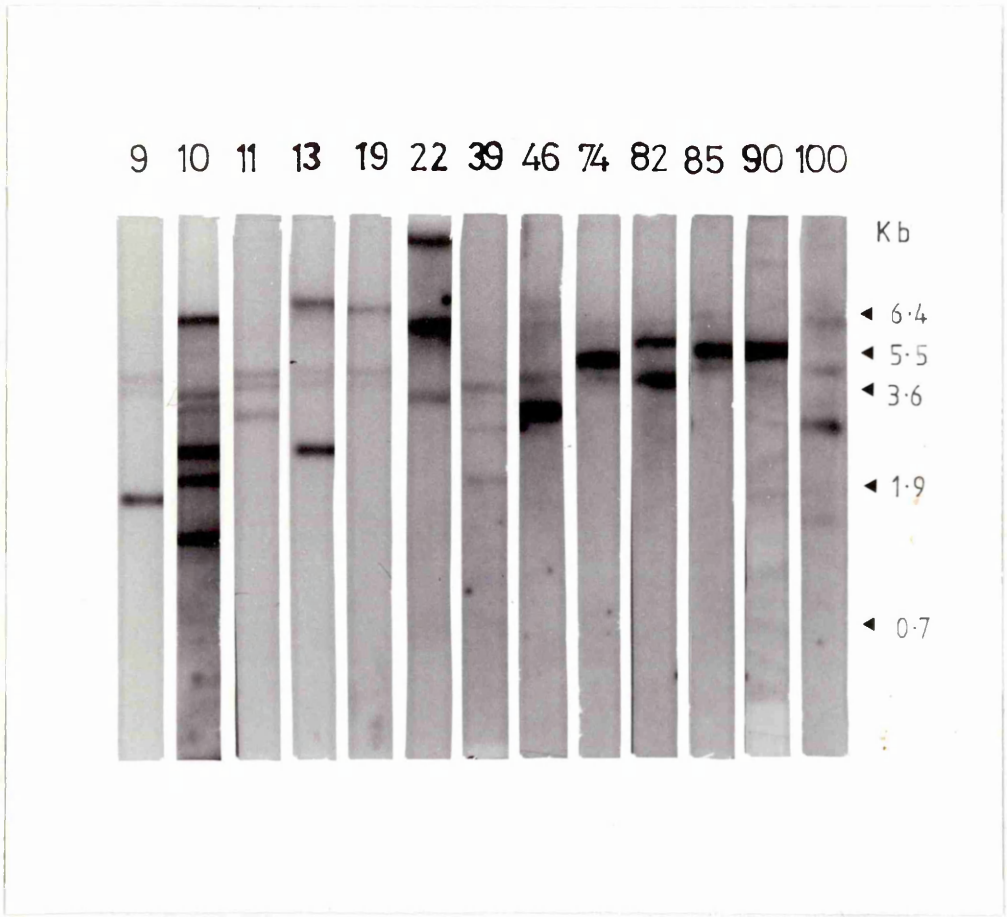


Fig.3.13. Southern blot analysis of the cDNA clones used in this study. Electrophoresis, blotting and probing of individual lanes containing 5ug of EcoRI digested yeast genomic DNA were done as described in the text. Number above each lane corresponds to the cDNA clone whose plasmid DNA was used as hybridization probe.

translated cDNA probes (Fig.3.13.). Most of thecDNA sequences appeared to be derived from single copy genes. However,cDNA 10 gave 4 major bands on the Southern blot(Fig.3.13). It is possible that the gene corresponding to cDNA 10 contains 4 Eco RI sites. However, unless this gene has introns, this explanation is unlikely since mRNA 10 is only 1250 bases long. (The lengths of the 4 major EcoRI fragments which hybridize with cDNA 10 are about 12 kilobases in total). Another possibility is that cDNA 10 was derived from a small gene family, in which case more than one gene may be transcriptionally active. Hence the mRNA half-life estimated using cDNA10 as hybridization probe may be an average of half-lives for several related mRNAs of similar length.

The Southern and northern analysis also suggested that all the cDNAs were derived from different mRNAs, since different DNA restriction fragments or mRNA species were detected with each cDNA. To confirm this, an experiment was performed in which each cDNA insert was cross-hybridized with the others. The cDNA inserts from each clone and the PYK genomic fragment from the PYK clone, were isolated, denatured and dotted on to nitrocellulose filters as described in Chapter 2. Identical filters were probed with nick-translated plasmid DNA from each clone (Fig.3.14.). None of the cDNAs cross-reacted with the other sequences or with the PYK sequence. Therefore, all the cDNA sequences were derived from different yeast mRNAs.

Finallly the lengths of the cDNA inserts were measured by digestion with PstI and agarose gel electrophoresis (Fig.3.15), The lengths of some of the cDNA inserts are greater

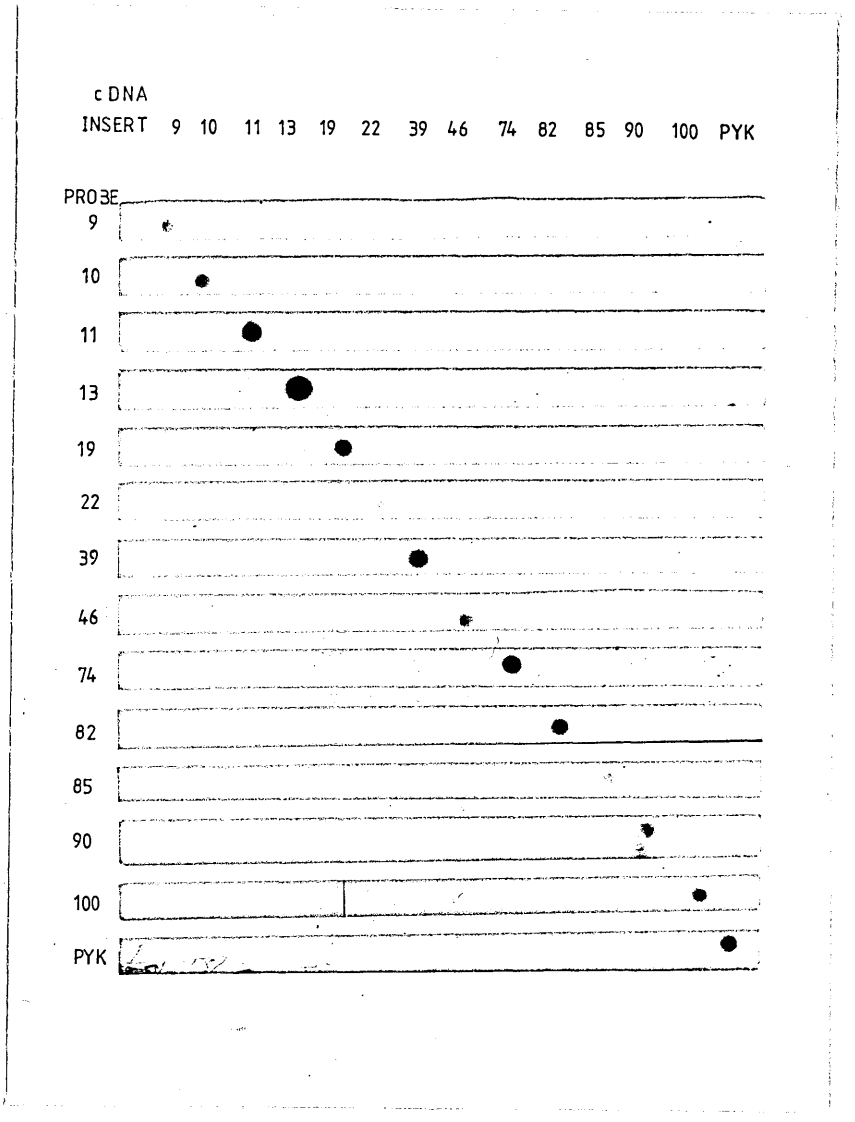


Fig.3.14. Autoradiograph showing the cross-hybridization pattern of 13 cDNA clones and the PYK genomic clone. Filters containing the purified PstI fragments from all the cDNA clones and the PYK genomic fragment were individually probed. Numbers along the top represent the plasmid cDNA from which the PstI fragment was derived. Numbers on the left corresponds to the plasmid cDNA used as probe.

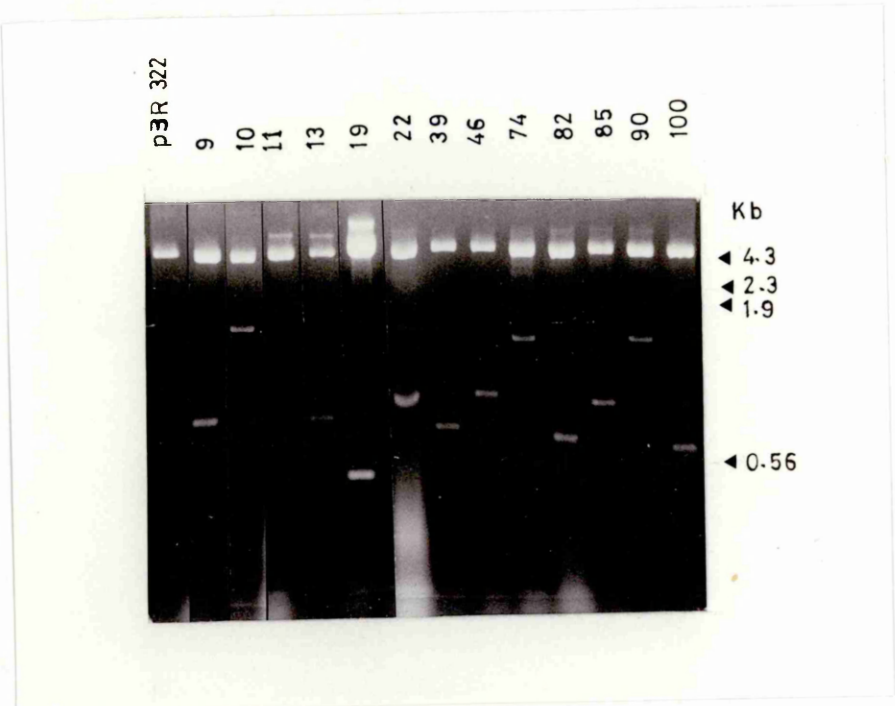


Fig.3.15. PstI digest of the plasmid DNA from the various cDNA clones used in this study. The number above each lane corresponds to the cDNA clone used.

than the mRNAs from which they derived (Table 3.1). In some cases (e.g. mRNAs 9,39 and 46) the discrepancies can not be accounted for by experimental errors in the measurements of cDNA and mRNA length, or by the G:C tails on the cDNA inserts. They may have arisen through the formation of artefactual cDNA molecules by reverse transcriptase (Fagan et al., 1980). Such increases in cDNA length have been reported previously during the cloning of chicken ovalbumin cDNA sequences (O'Hare et al., 1979) and fibronectin cDNA sequences (Fagan et al., 1980). However, the presence of the extra sequences did not appear to affect their use as hybridization probes.

3.3. DISCUSSION

In this study the mRNA:cDNA hybrid cloning procedure described by Wood and Lee (1976) and Zain et al. (1979) was used, since it is simple and less time consuming than other cDNA cloning methods. Previous authors reported that this method gives a frequency of transformation as low as 10^3 per microgram of mRNA:cDNA hybrid (Wood and Lee, 1976). However, I have obtained a frequency of 10^5 transformants per microgram of mRNA:cDNA hybrid. This is comparable to other methods in which double-stranded cDNA is used (Okayama and Berg, 1982). There may be a number of reasons for this discrepancy. Firstly, different vectors have been used in each study. While Wood and Lee (1976) used Col E1, pBR322 was used in this study. Secondly, in this study an E.coli strain which transforms at high frequency was

used in combination with efficient transformation procedures. Finally, the ratio of the cDNA insert to vector was carefully controlled in this study. The optimum molar ratio of 2:1 (vector:insert) was used to construct the library.

The attempt to isolate short and long half-life mRNA sequences by differential screening was not successful. This might be due to various reasons. The heat shock response obtained in this study may have been inadequate despite the appearance of presumed heat shock proteins after *in vitro* translation of 'heat-shocked' poly(A)+ mRNA. Alternatively, short half-life mRNAs may not have been degraded as rapidly as expected, after heat shock. However, it was not possible to increase the time of heat shock since it has been shown that yeast cells start to recover after 20 minutes and they start to return to normal patterns of protein synthesis (McAlister and Finkelstein, 1980). As described above, the half-life of mRNA 11 was much shorter than that expected from the differential screening. (See also Chapter 5.) It is possible that mRNA 11 was continuously transcribed during heat shock (Lindquist, 1981; Miller *et al.*, 1982). However, it is also possible that relative mRNA degradation rates change during heat shock and that the differential screening was based upon the incorrect assumption that rates of degradation remain constant. This needs to be tested experimentally.

To summarise, 13 cDNA clones containing sequences specific to 13 different mRNA species have been isolated from a yeast cDNA library by differential screening and by random choice. From Southern blotting it appears that most of the mRNAs correspond to single copy genes except mRNA 10. Each cDNA clone yields a single band on northern blotting and therefore, each is suitable for use in the analysis of mRNA stability in yeast.

CHAPTER 4

THE TECHNIQUE FOR mRNA HALF-LIFE ESTIMATION

4.1.Introduction

Once the decision is made to express a gene, the level of expression of the gene is normally dependent upon the steady-state level of the specific mRNA. The steady-state level of an mRNA depends not only on the rate of synthesis (i.e. transcription, processing and transportation into the cytoplasm), but also on the rate at which it decays and hence upon the factors that control that rate of decay. For example, in sea urchin embryos the steady-state levels of the so called 'house-keeping' mRNAs are thought to be determined primarily by their stability, because the steady-state levels of these mRNAs appear to correlate well with their half-lives rather than their transcription rates (Cabrera et al.1984). Thus differential mRNA stability plays a critical role in determining the rate of expression of a number of important genes.

The stability of mRNAs can be expressed either in terms of functional or chemical half-life. Functional half-life defines the rate of loss of the ability of the mRNA to serve as a template for protein synthesis. Chemical half-life, on the other hand is directly related to the presence of the mRNA sequence within the cell, whether it is biologically functional or not. Hence, theoretically, the chemical half-life is expected to be same or greater than the functional half-life for the same mRNA species. However, the average functional half-life for total mRNA in S.cerevisiae was estimated as 21±4 minutes (Tonnesen and Friesen 1973), whereas the equivalent chemical half-life for

total mRNA was estimated as 20 minutes (Kraig and Haber,1980). This discrepancy may possibly be due to the experimental errors resulting from different methods they have used in estimating the half-lives and it illustrates the technical difficulties that can be encountered in measuring mRNA half-life.

The functional half-life of mRNAs in S.cerevisiae have been measured using either a temperature sensitive mutant (ts 136) or transcription inhibitors such as lomofungin, daunomycin, or ethidium bromide. Tonnesen and Friesen (1973) used the mutant ts 136, which is defective in cytoplasmic RNA production at the restrictive temperature (37°C) , to estimate the average half-life of cytoplasmic mRNA. Chia and McLaughlin (1979) and Koch and Friesen (1979) have used the same mutant to measure individual mRNA half-lives. In these experiments, an exponential culture of the ts 136 mutant was shifted from 23°C to 37°C. Aliquots of the culture were then withdrawn at various times and the cells pulse labelled for 5-6 minutes with (^{35}S)-methionine. The proteins were then extracted and subjected to two-dimensional polyacrylamide gel electrophoresis. The rate of decrease in the levels of (^{35}S)-radioactivity in individual protein spots was used as a measure of the functional half-lives of their respective mRNAs. Kuo et al.(1973) have used lomofungin, and Tonnesen and Friesen (1973) used daunomycin and ethidium bromide to inhibit transcription in S.cerevisiae. Functional mRNA half-lives were determined by measuring the rate of incorporation of (^{3}H)-leucine into protein at various times after the addition of the inhibitors.

These estimates of functional mRNA half-lives werebased upon the assumption that the amount of protein synthesised using a particular mRNA sequence is proportional to abundance of the mRNA sequence present in the cytoplasm. However, this assumption may not be true, because the relative rates of translational initiation for different mRNAs are likely to change as the competition for initiation factors decreases (Lodish, 1974).

The chemical half-life of an mRNA can be measured either using incorporation kinetics or by decay kinetics. The principle involved in methods which use incorporation kinetics is that the incorporation of a radioactive precursor into a macromolecular species increases in a linear fashion until the synthesis and degradation of the labelled macromolecules approach equilibrium. The half-life of specific mRNAs can be estimated by measuring the hybridization of the labelled RNA with the appropriate DNA sequences at various times during the labelling period and thus measuring the time taken to reach equilibrium. Applying this principle, Greenberg (1972) measured mRNA half-life in HeLa cells and established mathematical procedures which have now been used by many other workers. Petersen et al. (1976) estimated the half-life of the total cellular poly(A)+ mRNA population in S.cerevisiae to be approximately 16 minutes using Greenberg's mathematical methods. Also, Hynes and Phillips (1976) obtained a half-life for total yeast mRNA of 17 minutes, and Kraig and Haber (1980) a half-life of 20 minutes. The half-lives of the URA3 and PPR1 mRNAs were estimated to be about 10 and 2 minutes, respectively (Losson and Lacroute, 1979; Losson et

al.,1983). The same research group has also estimated the URA1 mRNA half-life as 15 minutes (Pelsy and Lacroute, 1984). To obtain mRNAs of sufficiently high specific activity for the measurement of mRNA half-life using this procedure, yeast cultures are generally grown in minimal media. However, even then it is often hard to obtain sufficiently high specific activities to measure the half-lives of low-abundance mRNAs accurately. Furthermore, for the purposes of this study, the mRNA half-life measurements should ideally be performed using rich growth media to allow comparison of the half-life measurements with mRNA translation measurements. These are done in rich media.

Chemical mRNA half-lives can also be measured by decay kinetics. In this method, the cells are labelled with radioactive precursor (for example, (^{3}H)-adenine) to steady-state and then the labelled precursor is chased with non-radioactive precursor or the yeast is transferred to fresh medium (without radiolabelled precursor). The labelled mRNA is hybridized with the appropriate DNA sequences and the mRNA half-life is measured using the rate of decrease of radioactivity in the specific mRNA sequences during the chase period. Problems with this method lie in chasing the radiolabelled precursor from the intracellular pools using unlabelled precursor, due to the relatively high intracellular concentrations of mononucleotides in yeast (Bach et al., 1979). Theoretically, slow rates of removal of radiolabelled precursor from intracellular pools would result in overestimates of mRNA half-lives. Using a modification of this method, Bach et al. (1979) have estimated the URA3 mRNA half-life to be 10.5

minutes in yeast. This correlates well with the half-life of 10 minutes obtained for the URA3-18 mRNA using incorporation kinetics (Losson and Lacroute, 1979).

Yet another approach to the measurement of the chemical half-life of yeast mRNAs would be to use inhibitors of transcription. The principle here would be to block transcription with a suitable inhibitor and then to measure the rate at which specific mRNA sequences are cleared from the cytoplasm using hybridization procedures. This method had not been attempted in yeast prior to this study. The procedure would involve preparing total RNA at various times after inhibition of transcription and quantifying specific mRNA species in these RNA preparations using dot-blotting procedures. The rate of decay of the specific mRNA would yield its chemical half-life. It should be possible to measure the half-life of a number of mRNAs with ease and in a short time using this approach, since a variety of probes can be used to quantitate different mRNAs using the same RNA preparations. However, a drawback in this method is that the inhibitor may cause side-effects in the general metabolism of the cell. Also, it may be difficult to achieve complete inhibition of transcription in the absence of side-effects. However, it would be possible to draw valid conclusions about the relative half-lives of a group of mRNAs measured under the same experimental conditions using this approach. This technique was used to measure the half-life of c-myc mRNA in Burkitt's lymphoma cell lines using actinomycin D as the transcriptional inhibitor (Eick et al.,1985).

One of the main aims of this study is to measure the chemical half-life of a number of individual mRNAs as a basis for the investigation of factors which influence the half-lives of mRNAs in yeast. The last approach described above was chosen to measure mRNA half-lives since it is direct, and relatively simple. A number of inhibitors have been used to inhibit transcription in yeast. Tonnesen and Friesen (1973) used daunomycin and ethidium bromide as transcription inhibitors in S.cerevisiae, whereas 8-hydroxyquinoline was used in S.pombe by Fraser and Creanor (1974). Lomofungin has also been used in S.cerevisiae (Kuo et al.,1973). The mode of action of the phenazine antibiotic, lomofungin, involves chelation of the divalent cations required for activity of the RNA polymerases (Fraser and Creanor,1974). It has also been shown by these authors that the mechanism of inhibition of RNA synthesis by 8-hydroxyquinoline in S.pombe is also by chelation of divalent cations. Johnston and Singer (1978), reported that 1,10-phenanthroline inhibits RNA synthesis in S.cerevisiae. It was shown that the inhibition of RNA synthesis was due to the formation of an enzyme-zinc- phenanthroline complex (Auld et al.1976). Since the drug chelates zinc specifically, it was thought that it would have less effect on other cellular processes which require divalent cations such as translation. Furthermore, the drug is readily available. Hence phenanthroline was used in this study.

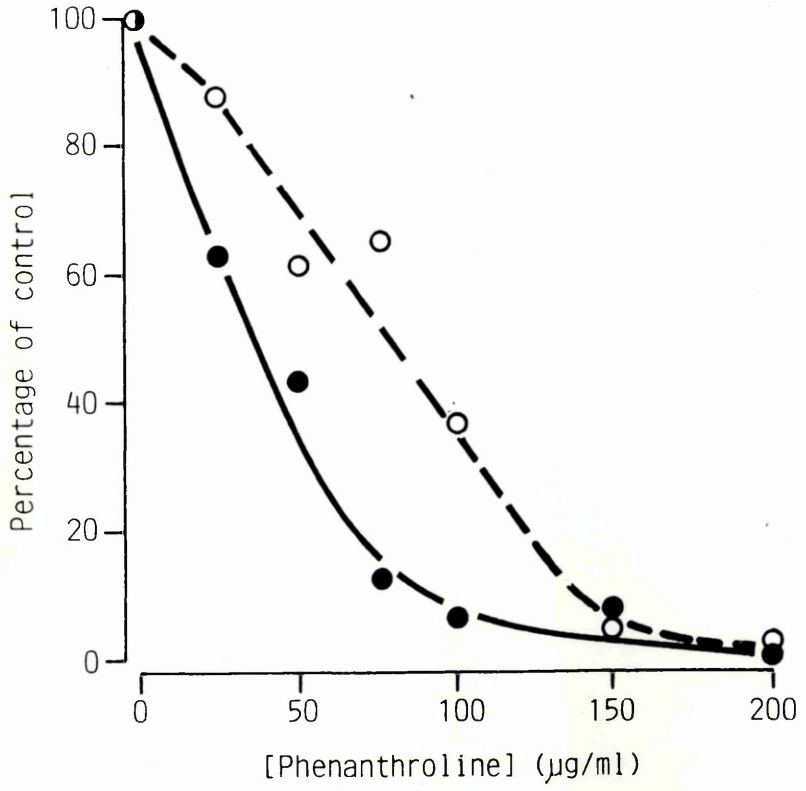


Fig.4.1. Effect of various concentrations of phenanthroline on RNA (●) and protein synthesis (O) at 7.5 minutes after drug addition in S.cerevisiae. All the values are expressed as percentages of the control values obtained using identical cultures to which only an equal volume of only ethanol was added.

4.2.Results

Preliminary experiments were performed to identify the optimal concentration of phenanthroline required to achieve maximum inhibition of transcription with minimum disruption of protein synthesis *in vivo* in *S.cerevisiae*. Various concentrations of phenanthroline (0,25,50,75,100,150 and 200 ug per ml of culture) were tested on identical exponentially growing cultures of yeast (DBY 746; A_{650}=0.45) at 30°C in YPG medium. The incorporation of (^{3}H)-uracil into 'cold trichloroacetic acid precipitable material' during a 5 minutes labelling period was used as an estimate of the rate of RNA synthesis (See Chapter 2). The rate of protein synthesis was estimated through the incorporation of (^{35}S)-methionine into 'hot trichloroacetic acid precipitable material' during a 5 minute labelling period. In these experiments (^{35}S)-methionine was used as the labelled amino acid because rapid saturation of the intracellular methionine pool occurs after the introduction of methionine to yeast cultures (Warner *et al*.1976). Fig.4.1 shows the effects of various concentrations of phenanthroline on RNA and protein synthesis. Maximum inhibition of transcription associated with minimum disruption to protein synthesis was observed between 75 to 100 ug of phenanthroline per ml of culture.

Another experiment was carried out to study the effect of phenanthroline on RNA and protein synthesis at a concentration of 100 ug per ml of culture at various times after the addition of the drug. Phenanthroline was added to an exponentially growing culture of yeast (A_{650} =0.45) to a final concentration

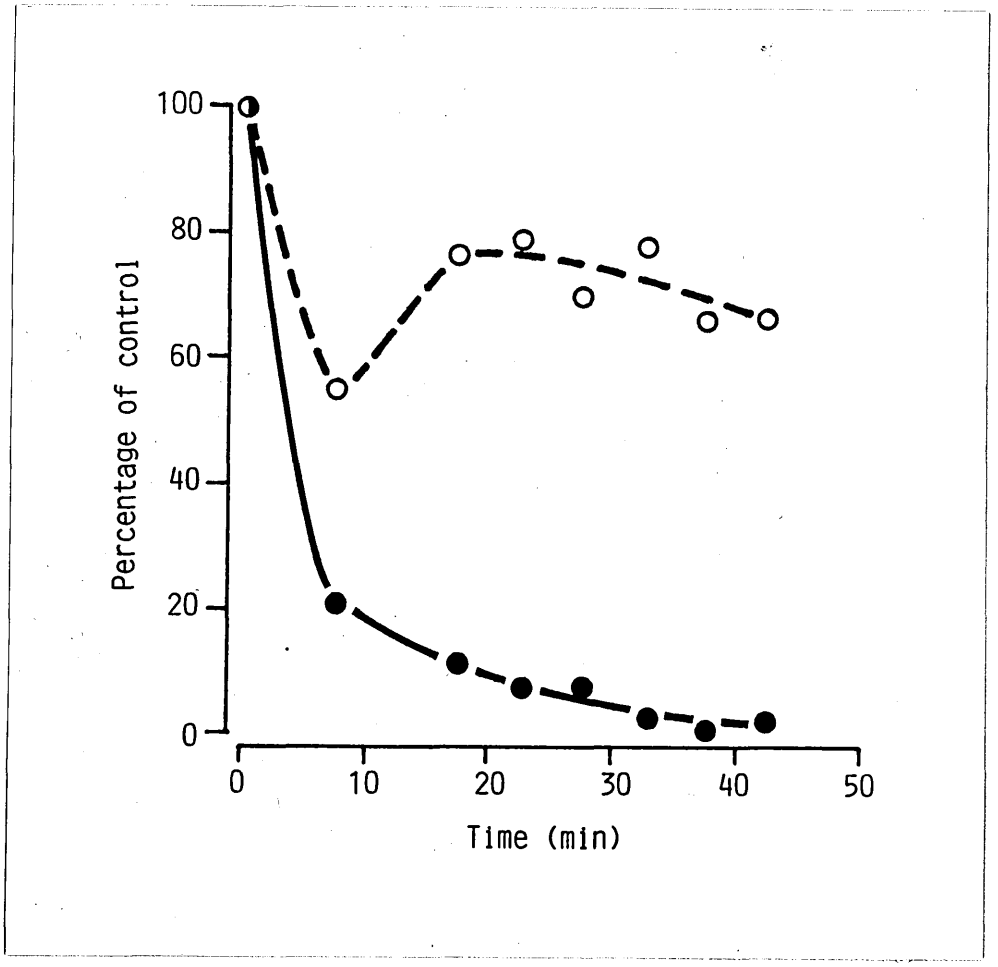


Fig.4.2. Effect of phenanthroline (100ug per ml) on RNA (●) and protein synthesis (O) in S.cerevisiae at various times after the addition of the drug. All the values are expressed as percentages of control values obtained using identical cultures to which only ethanol was added.

of 100 ug per ml of culture, and ethanol was added to an identical control culture. (Phenanthroline was added in ethanol.) Portions of the experimental and control cultures were pulse-labelled for 5 minutes with (^{3}H)-uracil at various times after the addition of phenanthroline (0,5,10,15,20,25,30,35,40 and 45 minutes). The incorporation of (^{3}H)uracil into cold trichloroacetic acid precipitable material was measured according to the procedures described in Chapter 2. A dramatic reduction in the rate of RNA synthesis relative to the control, was observed within 10 minutes. RNA synthesis was reduced to about 5% of the control by 30 minutes.The rate of protein synthesis was measured under identical conditions (Fig.4.2). Following a transient shock the cells regained their protein synthetic capacity. Finally, as expected, a gradual decrease in the rate of protein synthesis, was observed which was presumably due to the decay of mRNA after the addition of the drug.

It was possible that the effect of phenanthroline upon RNA synthesis could be due entirely to the inhibition of RNA polymerases I and III. This question was addressed by determining the proportion of poly(A)+ mRNA synthesised at various times after the addition of phenanthroline. An exponentially growing yeast culture was split into four equal portions and the growth continued. Phenanthroline (100 ug per ml of culture) was added to two portions, whereas ethanol was added to two control portions. (^{3}H)-uracil was added at 0 and 10 minutes after the addition of the drug or ethanol, and labelling continued for 10 minutes. After this, the pulse-labelled cells were harvested and RNA was

isolated from each of the four samples. The labelling periods were designed to measure the RNA synthesis 5 and 15 minutes after the addition of the phenanthroline or ethanol to the culture. Equal amounts of (^{3}H)-RNA (200,000 cpm per sample) were incubated with oligo(dT) paper from Amersham. The amount of bound radioactivity due to (^{3}H)-poly(A)+ RNA was calculated as percentage of the total (^{3}H)-RNA incubated with oligo (dT) paper. The results showed that 5 minutes after the addition of phenanthroline, poly(A)+ RNA synthesis was 14% of total RNA synthesis, compared to 21% in the control. After 15 minutes, poly(A)+ RNA synthesis was about 16% of total RNA synthesis in the presence of phenanthroline, compared to 17% in the control.These results demonstrate that polymerase II was inhibited to at least as great an extent as polymerases I and III. Hence phenanthroline is a suitable transcription inhibitor for use in the measurements of mRNA half-life in S.cerevisiae.

Johnston and Singer (1978) showed that lower concentrations of phenanthroline (20 ug per ml culture) inhibited the synthesis of 25S and 18S rRNA to a greater extent than the synthesis of mRNAs and tRNAs. Protein synthesis was essentially unaffected. The results of this study show that at higher concentrations (100ug per ml), total RNA synthesis could be reduced to a great extent and appear to have no direct, long term effect on translation. These results are similar to those obtained with 8-hydroxyquinoline at a concentration of 50ug per ml, in S.pombe (Fraser and Creanor,1974).

Fig.4.3. Decay of the mRNAs following the addition of phenanthroline to yeast cultures. Approximately equal amounts of RNA isolated at various times after the addition of phenanthroline (100ug per ml culture) was electrophoresed and northern blotting was performed as described previously. The blot was hybridized simultaneously with probes for the PYK mRNA, mRNA39 and mRNA90. The numbers at the top of the figure indicate the times after the addition of phenanthroline (in minutes) at which the RNAs were prepared. Numbers on the left show the positions of ribosomal RNA size stanadards and the positions of the three mRNAs analysed are shown to the right of the figure.

A northern blot was then performed on RNA isolated at various times after the addition of phenanthroline. Phenanthroline was added to an exponentially growing yeast culture and 100 ml portions of the culture were removed at various time-points after the addition of the drug. RNA was isolated from the cells at each time-point and northern blotting performed on 15 ug of each RNA preparation as described in Chapter 2. The blot was hybridized with nick-translated DNA from three clones simultaneously (39,90 and PYK; Fig.4.3).

The changing hybridization signals for each mRNA suggests that mRNA39 ,mRNA90 and the PYK mRNA all decay at different rates. These results suggest that these methods provide a suitable basis for the measurement of mRNA half-lives.

4.3. Discussion

The basis of a new and simple method to measure the half-life of mRNAs in yeast has been devised. This approach is different from the other techniques previously used to measure the chemical half-life of mRNAs in yeast, due to the use of non-radioactive RNA instead labelled RNA . Hence it is easy to make large quantities of RNA from multiple sample points and to prepare a large number of identical nitrocellulose filters containing the same RNA time-course. Thisallows rapid determination of many mRNA half-lives at one time, the half-life measurements being internally consistent . As no radioactive RNA

is used in the initial stages, radioactive contamination of laboratory equipment is minimal. This method can be used routinely, especially for comparative estimations of mRNA half-life.

However, no attempt has been made to confirm the validity of this approach by measuring the half-life of an mRNA whose half-life has been estimated previously by another method. The best candidate for a control mRNA would be the URA3 mRNA since its half-life has been measured previously using incorporation and decay kinetics (Bach et al. 1979; Losson and Lacroute,1979). However, these measurements were performed using minimal growth media, rather than the rich media employed in this study. Since growth rate affects mRNA half-life in E.coli (Nilsson et al.,1984) comparison of URA3 half-life using these different procedures may be invalid. Also, the yeast strain used in this study carries a mutation in the URA3 gene. It is likely that the half-life of the URA3 mRNA is altered in this strain.

CHAPTER 5

mRNA LENGTH AND HALF-LIFE

5.1. INTRODUCTION:

Eukaryotic mRNAs appear to decay exponentially (Greenberg, 1972; Petersen et al.,1976). This implies that new and old mRNAs have equal probablities of being degraded. Average mRNA half-lives have been estimated as 20 minutes in yeast S.cerevisiae (Kraig and Haber, 1980) and 10 hours in mouse L cells (Greenberg, 1972). However, individual mRNAs decay at different rates. In S.cerevisiae the half-lives for individual mRNAs range from about 3.5 minutes to 70 minutes (Chia and McLaughlin,1979; Koch and Friesen, 1979). Human beta and delta-globin mRNAs have half-lives of 16.5 and 4.5 hours, respectively (Ross and Pizarro, 1983). Also, the half-lives of actin and tubulin mRNAs in mouse L cells are 16-20 and 7 hours, respectively (Krowczynska et al., 1985). As described in Chapter 1, there are a number of reports in the literature which describe external factors which can affect mRNA stability. These include growth conditions, ploidy or strain variations in S.cerevisiae (Cooper et al. 1978),and hormones, viral infections or the differentiation stage of the cells in higher eukaryotes (Ross and Kobs, 1986). There are also reports pertaining to structural features that influence mRNA half-life in eukaryotes. Huez et al. (1974), Nudel et al.(1976), and Wilson et al., (1978) have shown that the 3'poly(A) tract plays a role in stabilizing mammalian cytoplasmic mRNAs, although there are also reports contrary to this view (Seghal,et al.,1978; Palatnik et al., 1980; Seghal and Sagar, 1980; Weissenbach et al., 1980).However, differences in

the 3' poly(A) tract are unlikely to be able to account completely for the differences in half-lives among poly(A)+ mRNAs.

Yeast mRNAs have a 5'-cap structures which has also been shown to stabilize mRNAs from exonucleolytic degradation (Furuichi et al., 1977). However, once again differences in the 5'-cap probably cannot account for the wide range of mRNA half-lives. It would therefore seem likely that important elements which determine mRNA stability are specified by internal features which lie between the 5'-cap and 3'-poly(A) tract. Such intrinsic features as sequence specificity, secondary structures and mRNA length, may delineate mRNA stability individually or in concert. These features may interact directly with the cellular apparatus for mRNA degradation, or may interact indirectly via translation and ribosome loading. However, it is technically difficultto study and hence to draw valid conclusions, about these individual parameters. For example, the theoretical analysis of secondary structure can be done using computer programmes, but these predicted secondary structures may not exist in vivo. However, chemical or biochemical probes may be used to test for their existence (Vary and Vournakis, 1983). If the problem of isolating sufficient quantities of a specific mRNA in the native form, or of studying a specific mRNA in vivo is overcome, the investigation of a link between the mRNA secondary structure and half-life would undoubtedly be rewarding.

The influence of mRNA length on mRNA stability is a contentious issue. Blundell et al. (1972) showed that there is no correlation between the length of the mRNA and its stability in E.coli. However, in eukaryotes there seems to be an indication that the size of the mRNA may be related to stability (Singer and Penman, 1973; Spradling et al., 1975; Berger and Cooper, 1975; Levis and Penman, 1977; Meyuhas and Perry 1979). The results presented here show that there is a complex but clear correlation between mRNA length and stability in S.cerevisiae.

5.2. Results

The half-lives of individual mRNA species were measured by dot blot analysis rather than by northern blots (See Chapter 2). Dot blots have been used successfully by Kafatos et al. (1979) for quantitative estimations of nucleic acids and have many advantages over northern blots. For example, it is possible to analyse triplicate or quadruplicate samples for many time-points on a single filter. Dot blotting is very convenient as a large number of identical filters can be prepared in a short time and stored for future use. Furthermore, it avoids the possible errors of uneven loading of samples to gels and the variable transfer to nitrocellulose filters sometimes observed in northern blots. It is also possible to cut each dot out from the filter and measure the radioactivity directly by scintillation counting. We found this to be more reliable and reproducible than densitometric scanning.

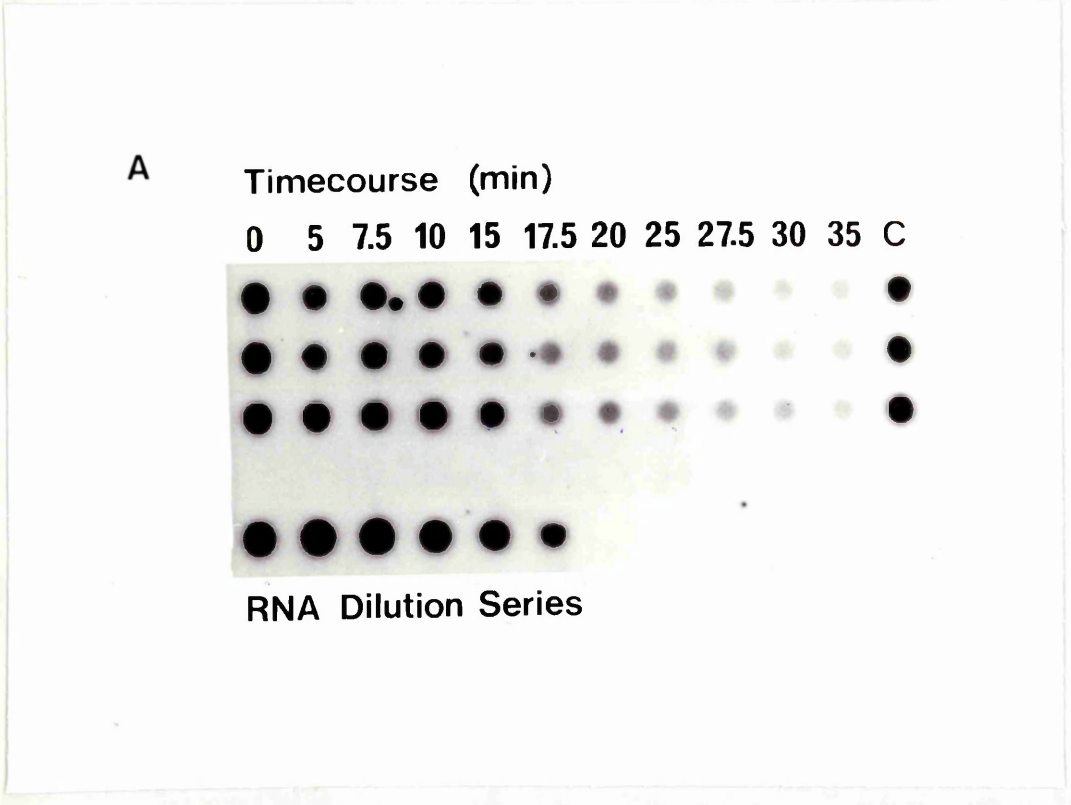


Fig.5.1. Autoradiograph of a dot blot (dots in triplicate) probed with the nick-translated cDNA plasmid90 . 'C' corresponds to the dots of a control RNA preparation to which equal volume of ethanol was added. Numbers above the dots the top correspond to time in minutes after the addition of phenanthroline at a concentration of 100ug per ml yeast culture.

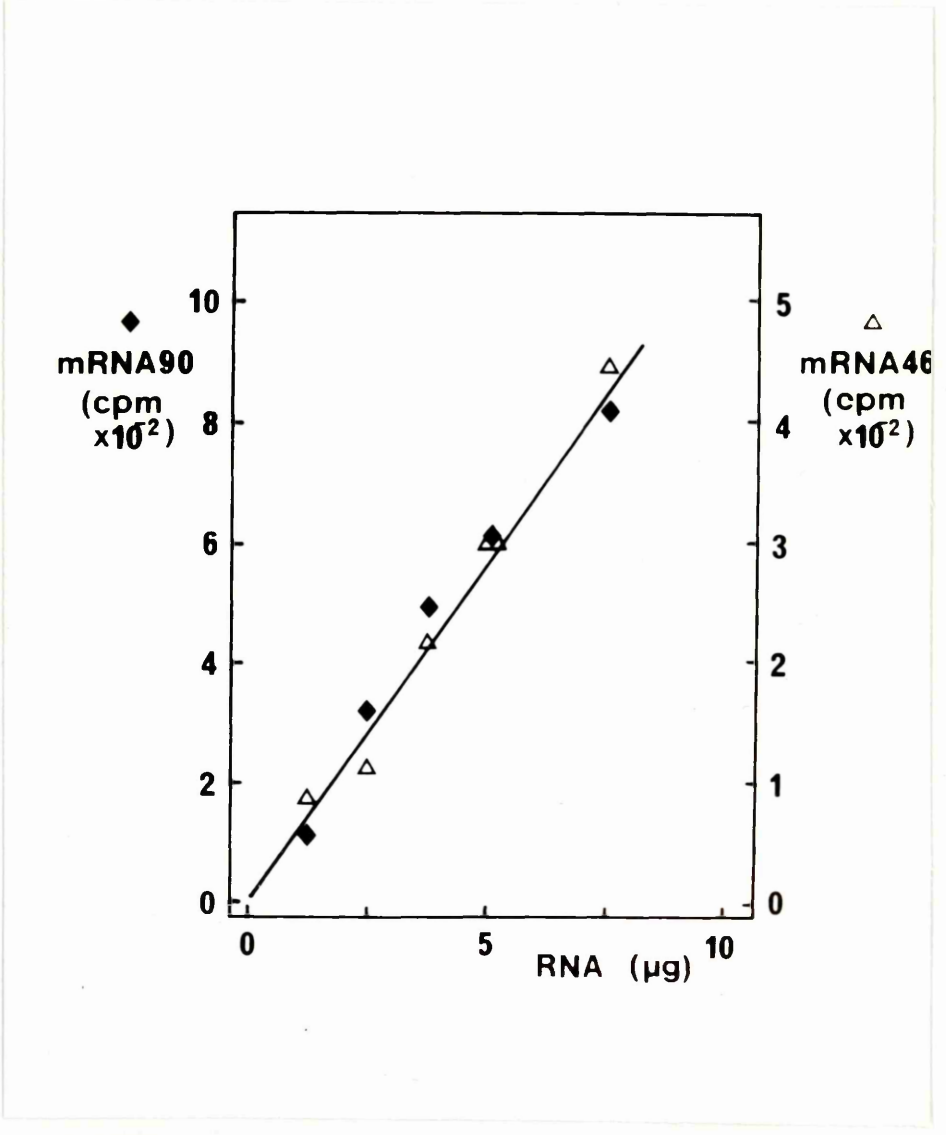


Fig.5.2. Graph showing the relationship between the radioactivity and the amount of RNA in the dilution series for mRNA90 (◆) and mRNA46 (△). The dots of the dilution series were cut and the radioactivity was measured by scintillation counting.

Identical nitrocellulose filters were prepared, each containing multiple dots of 5 ug RNA from the various time-points after the addition of the transcriptional inhibitor, phenanthroline (0, 5, 7.5, 10, 15, 17.5, 20, 25, 27.5, 30 and 35 minutes). The filters were probed separately with ^{32}P-labeled DNA from the 13 cDNA clones and the PYK genomic clone, which were described in Chapter 3. After hybridization, the filters were washed and autoradiographed to check for possible background radioactive contamination. Only filters with no detectable, or very low background levels of non-specific (^{32}P)-contamination (Fig.5.1) were used for mRNA half-life estimations. Each dot from the dilution controls on the nitrocellulose filters was cut out and the radioactivity was measured by scintillation counting. The radioactivity was compared with the amount of RNA dotted onto the nitrocellulose filter. Fig.5.2 shows that there is a linear relationship between the amounts of radioactive probe bound and mRNA dotted on the filter, over the range studied. Therefore, the amount of (^{32})P-DNA probe hybridized to each dot was a valid measure of the relative amount of that specific mRNA in the sample.

The hybridization pattern observed when probing a dot blot of an mRNA timecourse with (^{32}P)-cDNA90 is shown in Fig.5.1. The decrease in hybridization intensity of probe (cDNA 90) was shown to be proportional to the amount of mRNA 90 present in each individual dot. Therefore, it is possible to use the rate

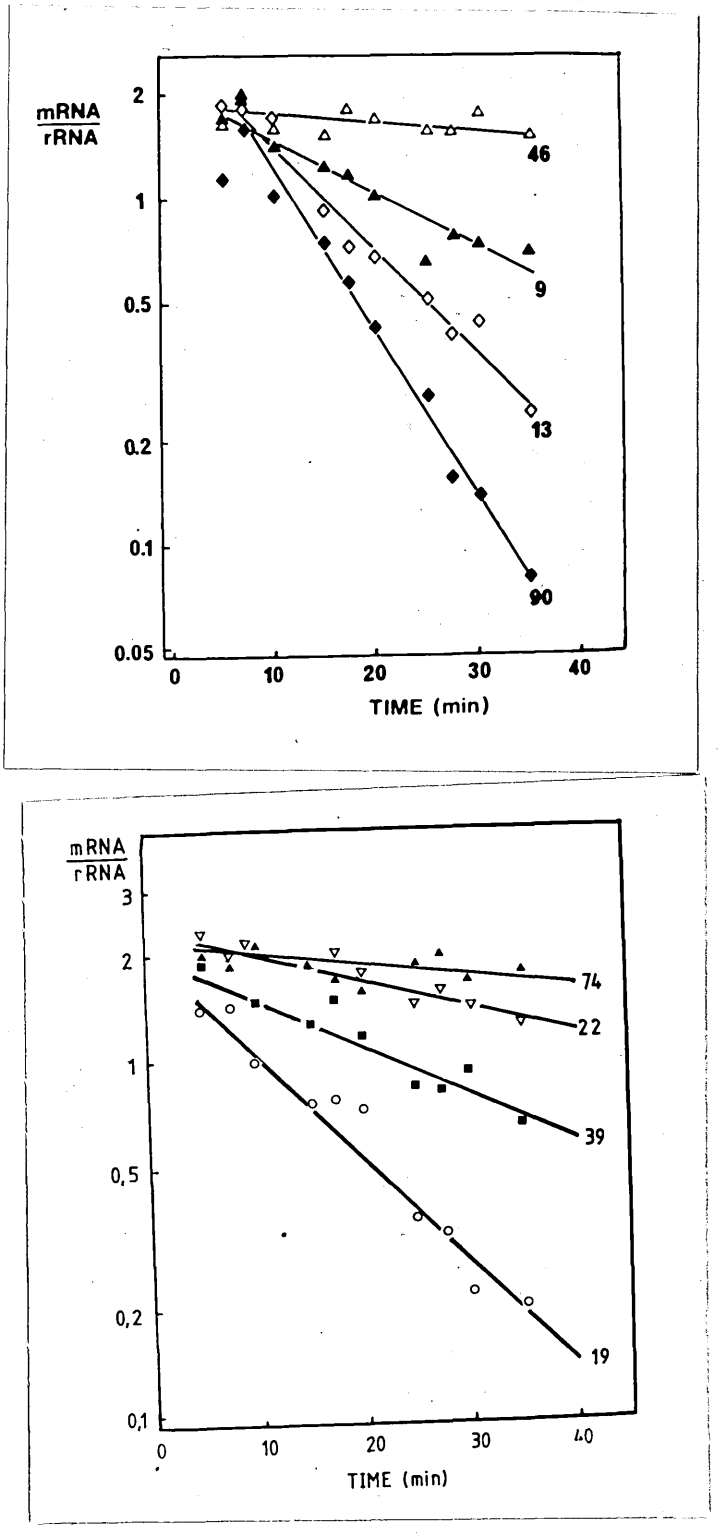
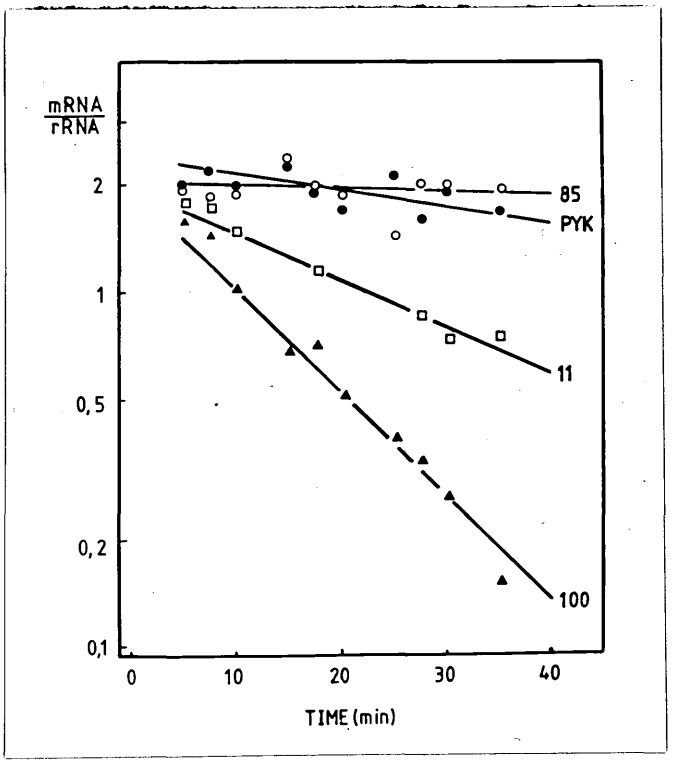
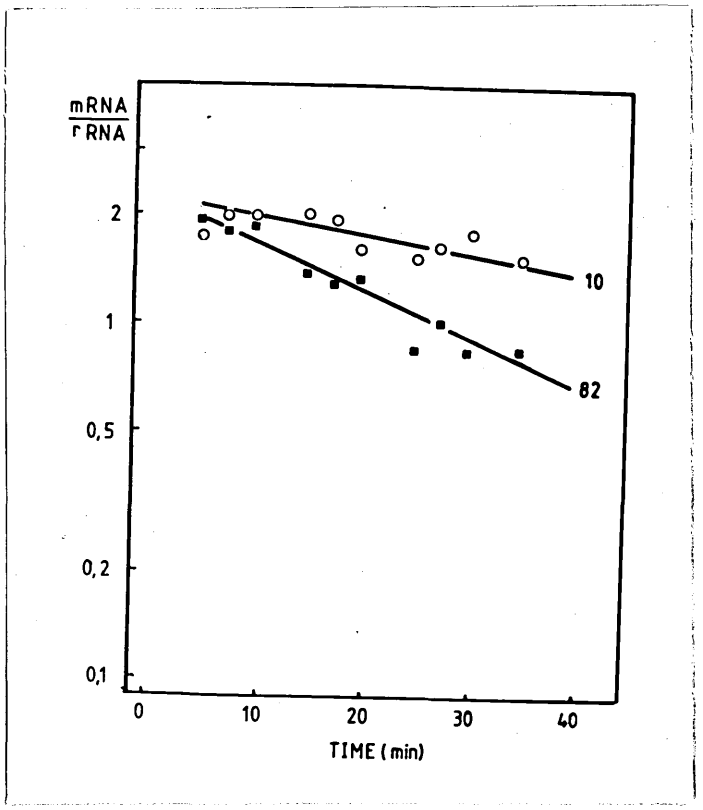


Fig.5.3. A,B,C and D show the semilogrithmic plot of the relative levels of mRNAs 9,10,11,13,19,22,39,46,74,82,85,90,100 and PYK versus time after the addition of phenanthroline at a concentration of 100ug per ml culture. The ratio between the mRNA and 18S rRNA specific activities were calculated as described in the text.

C


mRNA
rRNA
2
1
0,5
0,2
0,1
0
10
20
30
40
TIME (min)
85
PYK
11
100

D


mRNA
rRNA
2
1
0,5
0,2
0,1
0
10
20
30
40
TIME (min)
10
82

of decrease of the mRNA90 in the dots over the timecourse to estimate the half-life of that mRNA, after correction for differences in yield in RNA preparations throughout the timecourse.

Correction for differences in RNA yield between preparations in the timecourse was achieved by quantifying 18S rRNA in each preparation. This was done by probing filters, which contained roughly 5 ng of RNA from each time-point, with saturating levels of ^{32}P-labeled DNA sequences complementary to the 18S rRNA of S.cerevisiae. The filters were washed and autoradiographed, and radioactivity on each dot measured by scintillation counting as described above. The ratio of the values obtained for the specific mRNA and the 18S rRNA were then calculated giving 'corrected mRNA levels' for each time-point. Fig.5.3. graphically presents the relative rates of decay for mRNAs 9,10,11,13,22,39,46,74,82,85,90,100 and PYK. The corrected mRNA levels at each time-point were used to calculate statistically the rate of decay for the mRNA using the formula (Dixon and Massey, 1957):

$$b = \frac{\Sigma xy - \left(\frac{\Sigma x \times \Sigma y}{n}\right)}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

where b= slope of the line

y= log of ratio of mRNA to 18S rRNA specific activities

x= time in minutes after addition of drug

n= number of time points

cDNA CLONE	mRNA LENGTH (BASES)	HALF-LIFE (MINUTES)
9	440	16.5 ± 1.5
10	1,250	56.9 ± 6.0
11	700	18.0 ± 2.2
13	740	12.1 ± 1.0
19	550	15.3 ± 2.7
22	3,100	44.7 ± 2.4
39	380	18.3 ± 1.5
46	550	> 100
74	1,050	83.4 ± 9.2
82	370	22.3 ± 3.1
85	550	> 100
90	1,100	6.6 ± 0.67
100	500	10.4 ± 1.1
PYK	1,600	59.9 ± 7.8

Table 5.1. The length and half-lives of the mRNAs, used in this study. The mean half-life of the mRNAs are shown in minutes with standard error. The half-lives of mRNA46 and mRNA85 could not be measued accurately as they were more than 100 minutes.

RNA isolated at least from three independent timecourse experiments were used for each of the mRNA half-life estimations. From these values the mean half-life values were calculated. Table 5.1 shows the mean half-lives and standard error values for the 14 mRNAs used in this study. The half-lives ranged from 6.6 minutes for mRNA 90 to more than 100 minutes for mRNAs 46 and 85.

An important implication in using the ratio between the mRNA and 18S rRNA to correct for RNA yields is that the estimates of mRNA half-life are relative to the half-life of yeast 18S rRNA. Therefore, as the half-life of the mRNA approaches that of 18S rRNA it becomes difficult to measure the relative half-life accurately. Therefore, it was not possible to assign a meaningful half-life value to those mRNAs with relative half-lives exceeding 100 minutes (e.g. mRNA 46 and mRNA 85). Northern blotting demonstrated that the very stable mRNAs are definitely not homologous to the 18S rRNA.

A further limitation of this procedure for measurement of mRNA half-life must be considered. The half-life measurements may be slight overestimates since phenanthroline does not inhibit mRNA transcription completely. However,the half-life values for most of the mRNA species analysed in this study are well within the range of mRNA half-lives reported by earlier workers. For example, Koch and Friesen (1979) reported individual mRNA half-lives ranging from 3.5 minutes to 70 minutes. Also Chia and McLaughlin (1979) reported mRNA half-life values ranging from 4.5 minutes to 41 minutes. It should be noted that these were functional half-life values estimated from protein synthetic

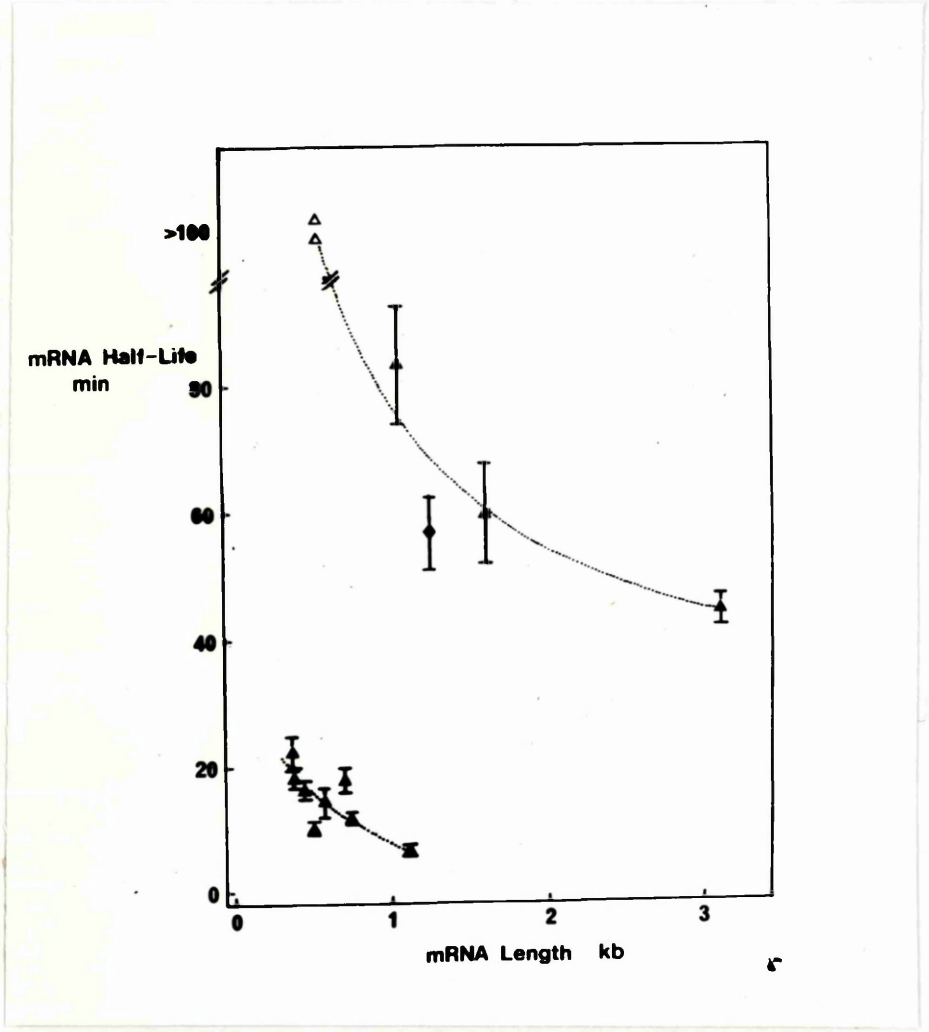


Fig.5.4. The relationship between mRNA half-life and mRNA length. All mRNAs are represented by ▲ except mRNA10 (◆), mRNA46 (△) and mRNA85 (△). Southern blotting analysis suggested that mRNA10 could be derived from a small family of genes (see Text). The half-lives for mRNA46 and mRNA85 are relatively long and therefore difficult to measure accurately. However, they have been included in the graph as they are consistent with the overall observation; both are 550 bases in length.

capacity. These are likely to be shorter than the chemical half-lives which have been measured in this study. Chen et al. (1984) reported that the half-life for the 3-phosphoglycerate kinase mRNA is between 70-80 minutes.

One of the aims of this investigation was to assess whether there is any correlation between mRNA length and half-life. Hence, the mRNA half-life values were plotted against length (Fig 5.4). The data clearly shows that, within each of the two groups of mRNAs, there is an inverse relationship between mRNA length and half-life. A similar relationship has been shown to exist in HeLa cells (Singer and Penman, 1973), mosquito cell lines (Spradling et al., 1975), in resting human lymphocytes (Berger and Cooper, 1975), in Drosophila (Levis and Penman, 1977) and in mouse L cells (Meyuhas and Perry 1979). In all of these cases, analysis of mRNAs on sucrose density gradients revealed that there was a general tendency for rapidly sedimenting mRNAs to be less stable than mRNAs which sedimented more slowly. The authors showed that this small, but significant difference in the sedimentation profile was not an artefact. Neither was this difference in length due to a reduction in the length of the 3'-poly(A) tract as the mRNA aged, nor to a limited reduction in the length of specific mRNA by an unknown mechanism. However, they only analysed gross mRNA population and not the behaviour of individual mRNAs. The existence of such an inverse relationship between length and stability is not unique to mRNAs.

A similar relationship has been found for proteins in higher eukaryotes (Dice et al., 1973), which indicates that length may influence the stability of these molecules.

The graph (Fig.5.4) also reveals that there are at least two populations of mRNAs in relation to mRNA length and half-life. One group contains mRNAs with relatively short half-lives and is termed 'unstable' while the other has mRNAs of relatively 'long' half-lives and has been called the 'stable' population. Within both populations longer mRNAs tend to have shorter half-lives. The existence of two populations of mRNAs in eukaryotes was suggested as early as 1973 by Singer and Penman. Later it was shown that similar populations of mRNAs existed in other eukaryotes such as Aedes (Spradling et al.,1975), in human lymphocytes (Berger and Cooper, 1975), Drosophila (Levis and Penman, 1977) and in mouse L cells (Meyuhas and Perry 1979). However, the basis for 'stable' and 'unstable' populations of mRNAs described in these reports was different from this study. In these previous reports the mRNAs were divided into two classes purely on the basis of their average half-lives, about 7 and 24 hours in HeLa cells, approximately 17 minutes and 24 hours in human lymphocytes, 1.2 and 20 hours in the mosquito cell line and 20 and 180 minutes in the Drosophila cell line. In contrast, in this study no distinct groups of mRNAs were observed in yeast; a continuous range of mRNA half-lives was measured. The

two stability classes were apparent only when mRNA length and half-life values were compared. To my knowledge, this comparison has not been made previously.

5.3. Discussion

The results presented here show that mRNA half-life is inversely related to its length in S.cerevisiae. For the first time this has been demonstrated for a set of specific mRNA sequences. The results are in agreement with the earlier reports in which gross mRNA populations were analysed in a wide range of eukaryotic organisms (Singer and Penman,1973; Spradling et al.,1975; Berger and Cooper,1975; Levis and Penman,1977; Meyuhas and Perry,1979). In contrast, the absence of a correlation between mRNA length and half-life has been shown in E.coli (Blundell et al.,1972).This may be due to a variety of reasons and might reflect the major differences in structural and functional organisation between prokaryotes and eukaryotes. For example, in prokaryotes transcription and translation are linked, and are not compartmentalised between nucleus and cytoplasm as in eukaryotes. Furthermore, the utilization of polycistronic mRNAs in prokaryotes is fundamentally different from eukaryotes in which monocistronic mRNAs are used almost exclusively (with the exception of some viral mRNAs). Also eukaryotic mRNAs have extensive post-transcriptional modifications, such as the 3'-poly(A) tract and the 5'-cap structure.

The inverse relationship between mRNA length and half-life suggests that the initial step in mRNA degradation might be a random event with an increased probablity of occuring on longer mRNAs. Longer mRNAs presumably present a larger target, or contains more nuclease-sensitive sites for an initial nucleolytic cut. (Exonucleolytic attack at the 3'-poly(A) tract, or removal of the 5' cap is unlikely to result in an inverse relationship between mRNA length and stability). A further conclusion can also be drawn about the mechanism of mRNA degradation in yeast, because hybridization techniques were used to measure chemical mRNA half-lives. The data suggest that mRNA sequences are degraded rapidly following the initial nucleolytic event. If mRNA sequences were not degraded rapidly, longer mRNAs would presumably take more time to be degraded than shorter mRNAs, but the opposite is observed in this study. Also slow degradation of mRNA sequences following an initial endonucleolytic cleavage would result in the accumulation of degradation intermediates *in vivo*. However, there was no detectable observation of hybridization to any RNA species other than the full length mRNA (e.g. mRNA90, mRNA39 and PYKmRNA; Fig.4.3). Similarly Rice and Roberts (1983) reported the absence of any intermediate breakdown products of actin or alpha-tubulin mRNAs in mouse L cells. Therefore, a mechanism of degradation for yeast mRNAs involving a rate limiting endonucleolytic cleavage followed by rapid degradation is consistent with these results.

Due to the existence of two populations of mRNAs it is possible for mRNAs of similar length to have very different half-

CLONE	mRNA LENGTH (BASES)	HALF-LIVES (MINUTES)	REFERENCE
ACTIN	1,300	76.6 ± 15	IAN PURVIS
PGK	1,500	70-80	CHEN ET AL (1984)
URA3	700	10.5	BACH ET AL (1979)
PPR1	2,900	2.0	FUCHS & LACROUTE (1983)
URA1	1,500	15.0	PELSY & LACROUTE (1984)
Rp1	1,310	13.1	KIM & WARNER (1983)
Rp29	620	14.0	KIM & WARNER (1983)
Rp39	660	12.0	KIM & WARNER (1983)
Rp73	480	16.0	KIM & WARNER (1983)

Table 5.2. mRNA length and half-lives. (results of other workers)

lives; for example, mRNAs 19 & 85, and mRNAs 90 & 74 (Table 5.1).

Similar situations exist in other higher eukaryotes. For example, human beta and delta-globin mRNAs are of the same length (1.6.kb) but have half-lives of 16.5 and 4.5 hours, respectively (Ross and Pizarro, 1983).

Most of the mRNAs used in this study are unknown. However, the half-lives of several yeast mRNAs have been measured previously (Table 5.2). When the half-life values for these mRNAs are compared with the limited information on mRNA length, they fit into the observed pattern reasonably well. Slight deviations from the observed pattern may be due to differences in the yeast strains, growth conditions and the techniques used to measure the half-lives of these mRNAs (Cooper et al., 1978). Most of the mRNAs analysed previously fit within the group of 'unstable' mRNAs (URA3, URA1, and the ribosomal proteins RP1, RP29, RP39 and RP73), but the PGK and actin mRNAs fit into the 'stable' group. There does not appear to be any clear functional relationship between the 'stable' and 'unstable' mRNA populations. The presence or absence of introns does not correlate with the 'stable' and 'unstable' groups, since the ribosomal protein mRNAs lie in the 'unstable' population whereas the actin mRNA belongs to the 'stable' group. (The half-life of the actin mRNA has been measured as 76.6±15 minutes by Ian Purvis of this laboratory.) Also since the PYK mRNA and PGK mRNA obviously belong to the 'stable' group, while the URA mRNAs belong to the 'unstable' population, mRNAs which encode

cytoplasmic enzymes can belong to either group. Therefore, the two mRNA populations are not due to differential cellular compartmentation.

The existence of two mRNA populations suggests that at least one other factor exists which significantly influences mRNA stability in yeast. (If mRNA length was the only determining factor, all mRNAs would fall within one population.) There are a number of candidates for this unknown factor and they include mRNA translation, differences in the 3'-poly(A) tract or 5'-cap structure, or the presence or absence of specific internal sequences or structures in yeast mRNAs.

A possible relationship between mRNA translation and stability was suggested as early as 1969 by Morse and Yanofsky. They demonstrated that mRNAs containing premature stop codons are degraded more rapidly than the equivalent wild-type mRNAs in E.coli. Similar observations have been made in a variety of eukaryotic systems, for example for human beta-globin mRNAs (Gorski et al.,1982), and the URA1 and URA3 mRNAs in S.cerevisiae (Losson and Lacroute, 1979; Pelsey and Lacroute, 1984). A simple explanation for these observations is that the ribosome may protect an mRNA from random endonucleolytic digestion during translation of the mRNA, and that mRNAs containing premature stop codons are not protected sufficiently against degradative enzymes. However, it is also feasible that a specific mechanism rapidly degrades any mRNAs which synthesise aberrant proteins. The possibility of a relationship between mRNA translation and stability in yeast is addressed in Chapter 6.

The presence or absence of a 3'-poly(A) tract cannot account for the division of yeast mRNAs into relatively 'stable' and 'unstable' populations since all the cDNA clones were constructed using poly(A)+ mRNAs. However, the possibility that a significant difference in the length of the poly(A) tract can account for the division of yeast mRNAs into 'stable' and 'unstable' population cannot be excluded. Nevertheless, this would seem to be unlikely.

In contrast, a fundamental difference in the 5'-cap could account for the observation. Two cap structures have been identified in yeast mRNAs; $m^7G(5')pppAp$ and $m^7G(5')pppGp$ (Sripati et al., 1976). Therefore, it is possible that the 'unstable' mRNAs carry one type of 5'-cap and the 'stable' mRNAs carry the other type. Some mRNAs have been sequenced and their transcriptional start sites mapped, but experimental error in the mapping techniques prevents absolute identification of the 5'-nucleotide. For example, the 5'-end of the PYK transcripts has been mapped to a G residue 33 nucleotides upstream from the translational start codon (Burke et al., 1983) but the margin of error (+/- 5 nucleotides) includes 5 A's and no further G's. Therefore, accurate 5' mapping must be performed on the mRNAs investigated in this study to test the possible involvement of the 5'-cap in differential mRNA stability. Nevertheless, if different cap structures were the basis for the division of yeast mRNAs into the observed populations, one would expect mRNAs with multiple transcriptional start sites (and therefore, a mixture of A and G residues at the 5'-cap) to lie between the two mRNA

populations. The stability of these mRNAs would depend upon their length and the proportion of sequences with each of the two types of 5'-cap.

Internal methylation of mRNA cannot cause the differential stability in yeast. In mammalian cells small mRNA molecules generally contain less internal 6'-methyl adenylate residues (m^6A) than do large mRNAs (Perry and Kelly, 1976) and m^6A is less prevalent in stable mRNAs (Friderici et al., 1976; Somer et al., 1978). Whether internal m^6A content is casually related to mRNA stability or not is still unclear. However, Sripati et al. (1976) have demonstrated that m^6A residues are not present in mRNAs from S.cerevisiae.

Specific structural signals on mRNAs are an important factor in mRNA stability and could provide the basis for the difference between the 'stable' and 'unstable' mRNA populations. Although the function of the 3'-untranslated region on eukaryotic mRNA remains obscure, a possible role in mRNA stability has been suggested (Littauer and Soreq, 1982). Sequence signals located in the 3'-untranslated region of the human beta and delta globin mRNAs are thought to determine the difference in their half-lives (Ross and Pizarro, 1983). The 3'-untranslated region has also been implicated in the stability of the hsp70 mRNA in Drosophila, since a deletion at the 3'-end increases the stability of this mRNA (Simcox et al.1985). The 3'-untranslated region of the yeast CYC1 mRNA appears to affect its stability (Zaret and Sherman, 1984). Mutant yeast strains carrying CYC1 alleles with altered 3'-termini contained different levels of CYC1 mRNAs and was this

presumed to be due to differences in the stabilities of the mRNAs carrying altered 3'-untranslated regions. However, no direct measurements of CYC1 transcription or CYC1 mRNA stability were made in the mutant strains to confirm this supposition. Interestingly, there did not appear to be a correlation between the apparent stability and the length for the mutant CYC1 mRNAs (Zaret and Sherman, 1984). It is tempting to speculate that the addition or deletion of signals in the 3'-untranslated region of the CYC1 mRNAs disrupted a normal inverse relationship between mRNA length and stability. Our observations have been made using normal yeast mRNAs; presumably additional factors come into operation to deal with abnormal mRNAs.

The 3'-untranslated regions of many genes have conserved sequences. For example, the actin mRNAs from birds, rodents and humans contain a conserved sequence in the 3'-untranslated region (Yaffe et al.,1985). Similarly the mRNAs for beta-nerve growth factor and the c-fos oncogene from divergent species also show a conserved sequences in the 3'-untranslated region (Yaffe et al., 1985). Even though the mRNAs encode similar proteins, one would expect the 3'-untranslated regions to have diverged significantly. Therefore, the conservation of sequences within these regions would suggest that they might have some functional significance, possibly in mRNA stability. More recently, a conserved sequence has been reported in the 3'-untranslated regions of a large number of mRNAs which code for various structurally unrelated, but functionally related proteins like, tumour necrosis factor (TNF), interleukin (IL), interferon (IFN),

granulocyte-macrophage colony stimulating factors (GM-CSF) in humans and in mouse (Caput et al.,1986). The mRNAs for all these proteins contained the consensus sequences UUAUUUAU in the 3'-untranslated region. Subsequently, it has been shown that the 51 nucleotide, A:U rich sequence from the 3'-untranslated region of the human GM-CSF mRNA is involved in mRNA destabilization (Shaw and Kamen, 1986) The sequence was introduced into the rabbit beta-globin mRNA which normally has a half-life of approximately 17 hours. As a control, another sequence of the same length, but having 14 G's and C's (interspersed with the A's and U's) was also introduced into the beta-globin mRNA sequence. The half-life of the mutant beta-globin mRNA containing A:U rich sequence was drastically reduced (approximately 30 minutes) compared with those of the mutant beta-globin mRNA with the control sequence containing G's and C's and the wild-type beta-globin mRNA (approximately 2 hours). This low level of beta-globin mRNA brought about by the insertion of the A:U rich sequence was not due to a reduced rate of transcription.

The 5'-untranslated region has been shown to play a role in stabilizing some viral , bacterial and eukaryotic mRNAs. For example, Gorski et al., (1985) showed that the stability of the bacteriophage T4 gene 32 mRNA is influenced by sequences in the 5'-leader sequence. The 70 nucleotide sequence, which has been implicated in stabilizing the gene 32 mRNA, is capable of folding into a secondary structure which is located just upstream from the initiation codon. The mRNA of internal protein III (IP III) from bacteriophage T4 is more stable than most of the other phage

transcripts (Kirsch et al., 1977). It may be significant that this mRNA is also capable of forming a secondary structure. It is attractive to suggest that this structure might be involved in stabilizing the mRNA. von Gabain et al. (1983) showed that the region containing the first 200 nucleotides of the 5'-leader sequence is the most stable part in the ompA mRNA of E.coli. It has also been shown that the ompA mRNA is stabilized by the 5' leader sequence (Green and Inouye, 1984). These authors showed that shortening of the 5'-untranslated region results in a reduced level of the ompA mRNA, due primarily to a decrease in the stability of the mRNA rather than to reduced rates of transcription. They also suggested that the high potential for secondary structure formation within the 5'-untranslated sequence may stabilize the mRNA. Though these reports show that the 5'-untranslated region can influence the stability of bacteriophage T4 and E.coli mRNAs, the mechanism by which this is achieved remains unclear. It is possible that secondary structure formation in the 5'-untranslated region protects the mRNA from 5'-exonucleolytic attack.

Recently, Belasco et al. (1986) showed that in E.coli the 5'terminal 147 nucleotide segment of the stable ompA mRNA (half-life 15 minutes) has a stabilizing effect. When this segement was fused to the unstable bla transcript (half-life 3 minutes) the half-life of the latter increased 3-5 fold, suggesting that this 147 nucleotide segment contains structural determinants of mRNA stability.

In eukaryotes, the 5' untranslated region has been shown to influence the stability of some mRNAs. Eick et al. (1985) showed that in Burkitt's lymphoma cell lines, the 5'-untranslated region of the c-myc gene mRNA has a destabilizing effect on the mRNA as a whole. The removal of the first 5'-exon, which does not contain any coding region, stabilizes the truncated mRNA. The mechanism by which the 5'-untranslated region influences the stability of the c-myc mRNA remains unclear.

CHAPTER 6

mRNA STABILITY AND TRANSLATION

6.1. Introduction

Messenger RNAs are involved in protein synthesis and hence interact with ribosomes and the protein synthetic factors. During protein synthesis each ribosome moves along the mRNA, covering about 30 nucleotides of an mRNA molecule at any one time (Lewin, 1983). To a certain extent, the ribosome may protect an mRNA from digestion by ribonucleases. Thus a relationship might exist between mRNA stability and ribosome loading.

A relationship between mRNA stability and ribosome loading has been shown in prokaryotes (Morse and Yanofsky, 1969; Morse et al., 1969). These authors demonstrated that in E.coli, mRNA degradation follows closely behind the last translating ribosome and occurs in the same direction as translation. These results indicated that the ribosome does at least partially protect an mRNA from degradation. Also Craig (1972) and Levinthal et al. (1963) showed that E.coli mRNAs are stabilized under conditions in which ribosome translocation is arrested. If ribosomes protect an mRNA from degradation, ribosome releasing agents should bring about accelerated decay of the mRNA. Varmus and Perlman (1971) have demonstrated that puromycin increases the rate of the decay of the lac mRNA in E.coli. Furthermore, Gupta and Schlessinger (1976) showed that in E.coli, mRNA degradation rates changed as the rates of ribosome translocation varied on the mRNA. Hence it has been proposed that ribosomes might limit the rate of a 5'-exonuclease activity, and thus

protect the mRNAs from degradation (Morikawa and Imamoto, 1969; Morse et al., 1969; Craig, 1972; Gupta and Schlessinger, 1976).

A similar relationship between translation and mRNA stability has been observed in eukaryotes. Singer and Penman (1972) showed that in HeLa cells, mRNAs decayed at increased rates in the presence of polysome disaggregating agents such as puromycin and fluoride ions. They suggested that mRNA stability is related to translation and that the release of ribosomes from the mRNA results in rapid mRNA decay. Trichodermin, an inhibitor of elongation and termination during protein synthesis in Saccharomyces cerevisiae, causes an increase in mRNA half-life (Wei et al., 1974). Similar results have also been obtained by Cooper et al. (1978). This increase in mRNA half-life might have been due to increased ribosome loading caused by decreased rates of elongation and termination relative to initiation of translation. However, these assumptions were not tested in vivo by analysis of polysomes. An alternative explanation would be that trichdermin directly inhibits mRNA degradative enzymes.

The ribosome protection model was later supported indirectly by the work of Losson and Lacroute (1979), who isolated URA3 amber mutants of S.cerevisiae and measured the half-lives of wild type and mutant URA3 mRNAs. They showed that the position of the nonsense mutations in the gene affected the stability of the corresponding mRNA. When the mutation was located near the 5'-end of the URA3 mRNA (URA3-3), undetectable levels of URA3 mRNA were observed. In contrast, when the nonsense mutation was positioned towards the 3' end of the mRNA (URA3-18),

levels of URA3 mRNA similar to that of the wild type were seen. The half-life of the URA3-3 mRNA was estimated as approximately 2 minutes, whereas that of the URA3-18 and wild type mRNAs were about 10 minutes.

Pelsy and Lacroute (1984) obtained similar results with URA1 mRNA mutants in S.cerevisiae. The half-life of the wild type URA1 mRNA was estimated as 15 minutes, but the half-life of an URA1 mRNA which had an ochre mutation at the 5' end of the coding region was about 1.5 minutes. In contrast, the half-life of an URA1 mRNA which contained an ochre mutation at the 3' end of the coding region was similar to that of the wild type URA1 mRNA.

Chang and Kan (1979) and Gorski et al. (1982) have reported that a human beta-thalassemia is caused by a nonsense mutation at the 17th codon in the beta-globin mRNA. The low levels of beta-globin mRNA in these patients might be due to either or both of the following. Beta-globin mRNA may be synthesised at abnormally low levels. Alternatively, degradation of the beta-globin mRNA might be increased, since a large part of the beta-globin mRNA would not be covered by ribosomes and would therefore be exposed to nuclease degradation.

The ribosome protection model in eukaryotic organisms is based upon analyses of mutant, not wild-type mRNAs. Therefore, alternative explanations exist for the observations. It is possible, for example, that there is a mechanism for the recognition and removal of mRNAs which encode aberrant proteins. If this were the case, URA3-3 mRNA would be degraded because it encodes a severely truncated protein rather than the mRNA being

susceptible to attack by ribonucleases because it is not protected by ribosomes. This mechanism of mRNA degradation would be an extension of the known degradative apparatus which clears the cell of the abnormal proteins (Goldberg and Dice, 1974).

One of the primary aims of this investigation was to investigate the relationship between mRNA stability and ribosome loading and hence to assess the validity of the 'ribosome protection model' in the yeast, S.cerevisiae. In general, if ribosome loading influences mRNA stability, long half-life mRNAs should carry relatively more ribosomes in vivo than short half-life mRNAs. An easy and efficient approach to this problem is to analyse the polysome profiles of a range of specific mRNAs. Estimates of 'ribosome loading' can be made from polysome profiles and these can be compared with the half-lives of these mRNAs.

6.2. RESULTS

The ribosome loading of individual mRNA species was estimated by combining polysome gradients and hybridization techniques. Forthe preparation of polysomes, cell lysates have been prepared either by breaking open yeast cells with glass beads (Kraig and Haber, 1980) or by gently lysing yeast sphaeroplasts (Hynes and Phillips,1976). The latter methodwas used in this study for three reasons. Firstly, while gentle lysis of sphaeroplasts is not likely to disrupt the polysomes, shearing could occur during cell breakage with glass beads. Secondly, it

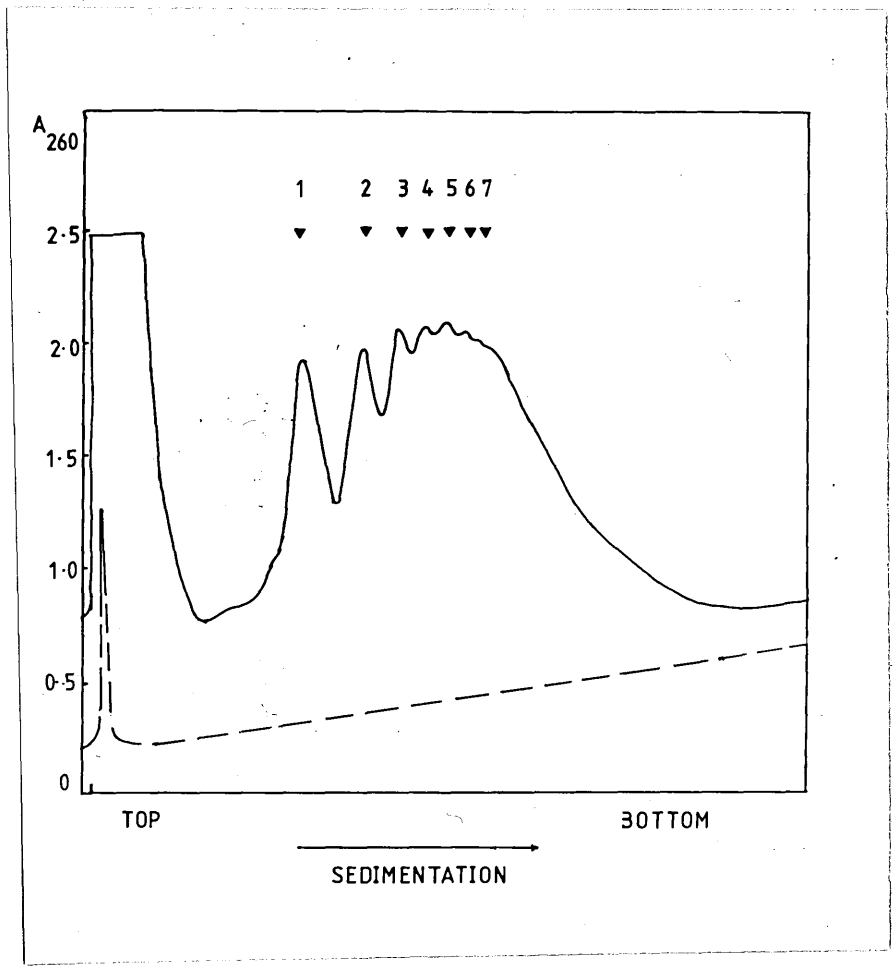


Fig.6.1. A_{260} polysome profile of S.cerevisiae. The polysome gradients were obtained as described in Chapter 2. The direction of sedimentation is shown by the arrow. Positions of the polysomal peaks are indicated by (▼). Broken line at the bottom represents the background UV absorption by sucrose.

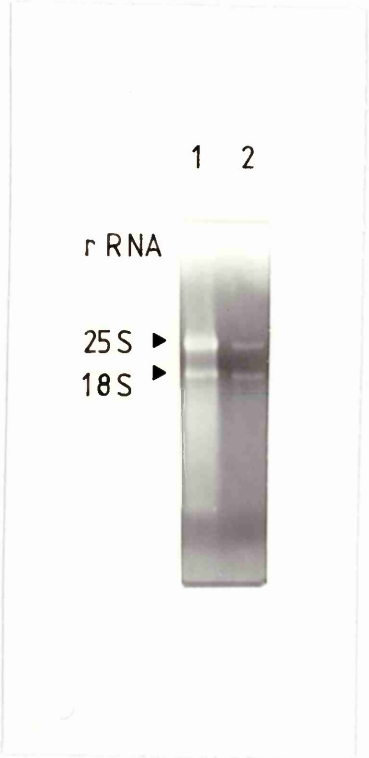


Fig.6.2. Agarose gel electrophoresis of RNA from fractions of the polysome gradient. RNA from various fractions were pooled and electrophoresed through 1% (w/v) agarose in TBE buffer. Positions of the ribosomal RNAs in lane 1 and 2 are indicated.

is not known whether the mRNAs used in this study code for intracellular or secreted proteins. While membrane-bound polysomes are likely to be lost during the glass bead method, both membrane-bound and free polysomes are obtained after gentle lysis of sphaeroplasts with the appropriate detergents (Hynes and Phillips, 1976). Finally, polysome profiles obtained using sphaeroplasts were more reproducible than those obtained using glass bead method. It has been shown previously that sphaeroplasts are physiologically similar to intact yeast cells (Hutchison and Hartwell, 1967).

Preparation of sphaeroplasts, sucrose density gradient centrifugation of polysomes and fractionation of the gradients were done according to the methods described in Chapter 2. For successful isolation of polysomes it was necessary to inhibit ribonuclease activity, since even a single break in the mRNA strand is sufficient to dissociate a large polysome into smaller fragments. However, no ribonuclease inhibitor was added during the preparatory steps to avoid possible side-effects on the polysomes. Instead, utmost care was taken to make the apparatus and buffers RNase-free and to perform the experiments as rapidly as possible.

Fig.6.1. shows the A_{260} profile of yeast polysomes after sedimentation on sucrose gradients. Routinely, polysomes containing at least five to six ribosomes could be resolved on the gradients. RNA from various fractions were pooled and electrophoresed through agarose gel to test whether the RNAwas intact(Fig.6.2). This was confirmed by performing a northern blot

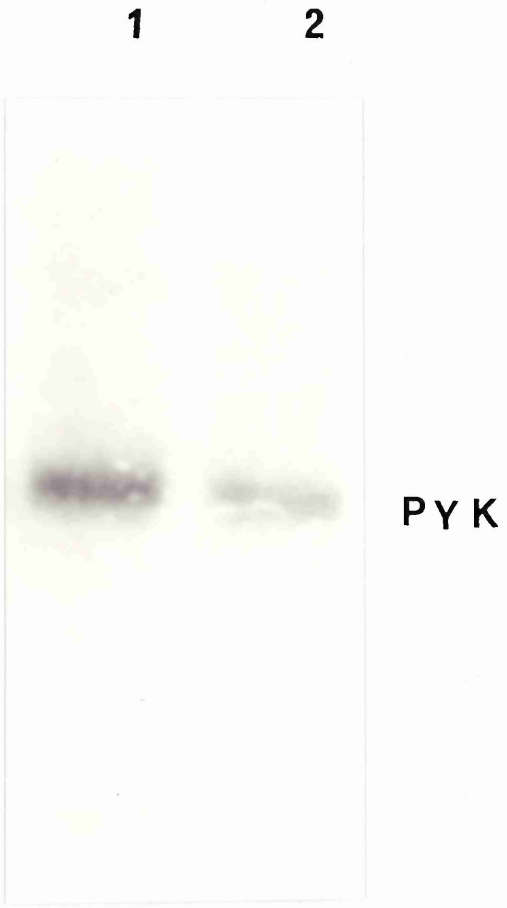


Fig.6.3. Northern blot analysis of the RNA from fractions of the polysome gradient. RNA from the fractions of the polysome gradient was electrophoresed, blotted and probed with nick-translated PYK DNA sequences as described previously. The position of PYK mRNA on lanes 1 and 2 is indicated.

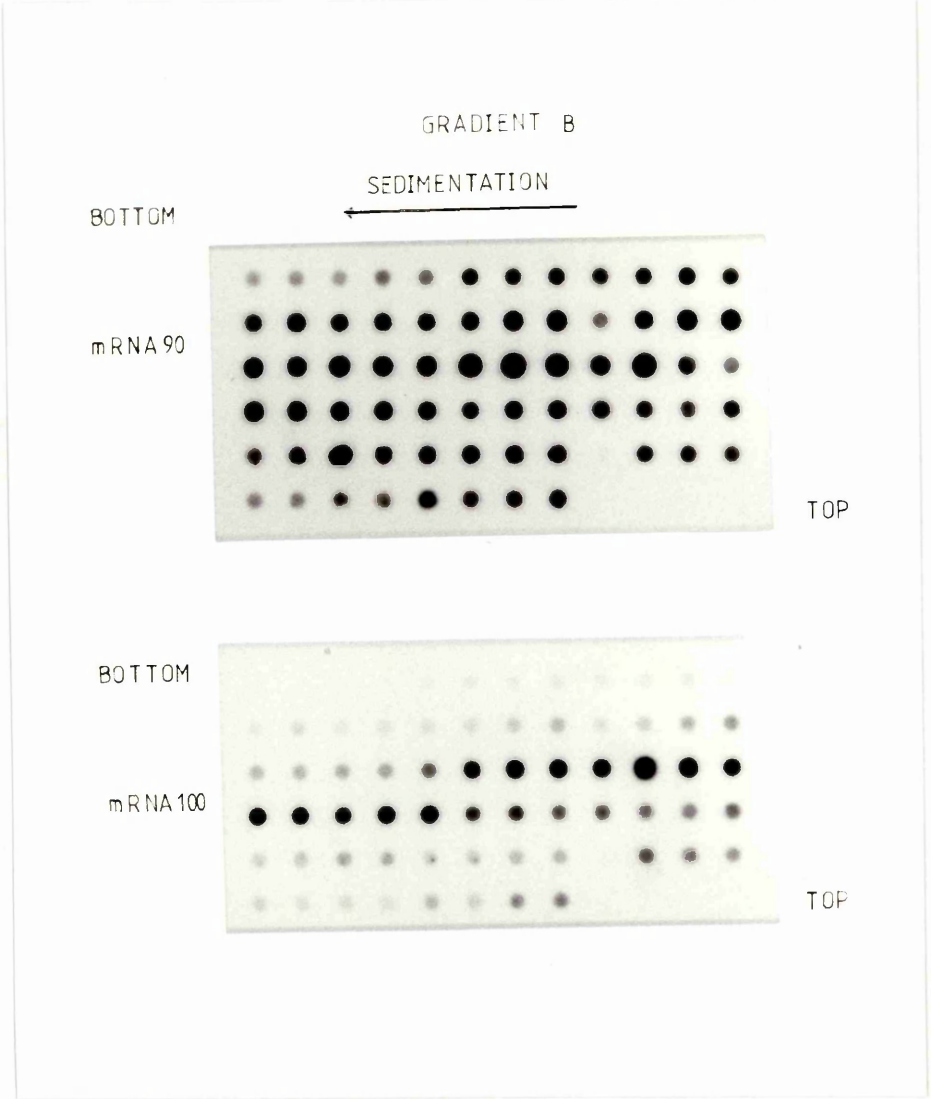


Fig.6.4. Autoradiograph showing the distribution of mRNA90 and mRNA100 across the polysome gradient. RNA from each of the fractions of the gradient was dotted onto the nitrocellulose filter and probed separately by nick-translated DNA from cDNA90 and cDNA100. Arrow shows the direction of sedimentation.

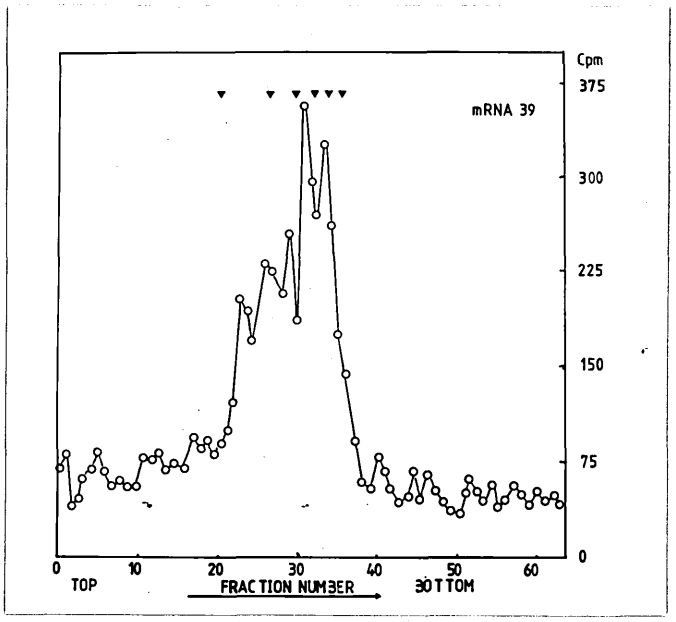
on RNA from the various fractions and probing the blot using nick-translated PYK DNA. Fig.6.3. shows that the PYK mRNAwas intact, confirming the efficacy of the precautions against ribonucleases. Also, there was no detectable shearing of polysomes.

The distribution of individual mRNA species on the polysome gradients was determined by hybridization techniques (Chapter 2). Since this method is sensitive it is possible to analyse mRNAs of low abundance, and to compare a number of mRNA species on the same gradient. Fig.6.4. shows the autoradiograph after probing a dot blot of fractions from a polysome gradient with nick-translated cDNA90 and cDNA100. The graphs in Fig.6.5 and Fig.6.6 were obtained by measuring the radioactivity in individual dots from blots similar to Fig.6.4. The distributions of mRNA74 and mRNA90 is illustrated in Figs.6.6.

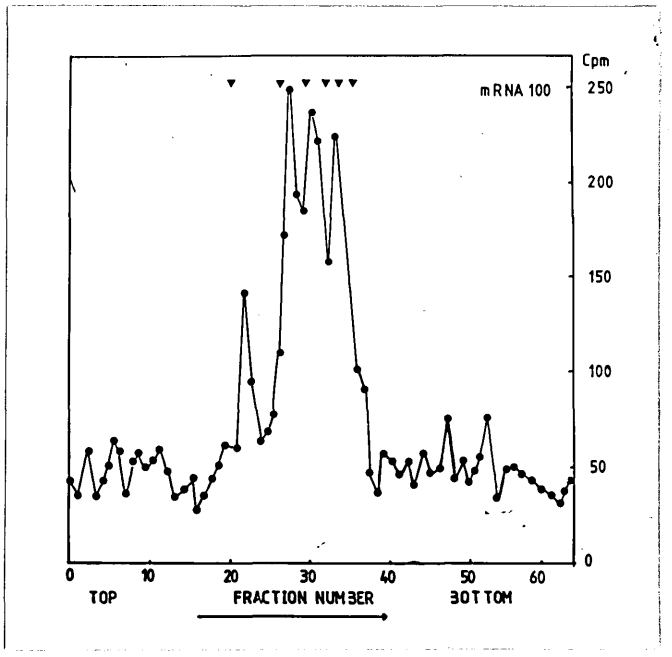
By comparing the distribution of a specific mRNA with the A_{260} polysome profile obtained for the same gradient, it was possible to determine the relative proportions of the mRNA in particular portions of the gradient (e.g. post-polysomal, monosomal, and various polysomal fractions). This was done by cutting and weighing the graphs (Palmiter et al., 1970). The results for the mRNAs analysed are summarized in Table 6.1. All the mRNA species analysed in this study have been found in association with polysomes.

It is not possible to make direct comparisons of the mRNA distributions between gradients, because slight variations in the physiological state of the cells can result in alterations

A


Cpm
375
300
225
150
75
0
mRNA 39
0
10
20
30
40
50
60
TOP
FRACTION NUMBER
BOTTOM

B


Cpm
250
200
150
100
50
0
mRNA 100
0
10
20
30
40
50
60
TOP
FRACTION NUMBER
BOTTOM

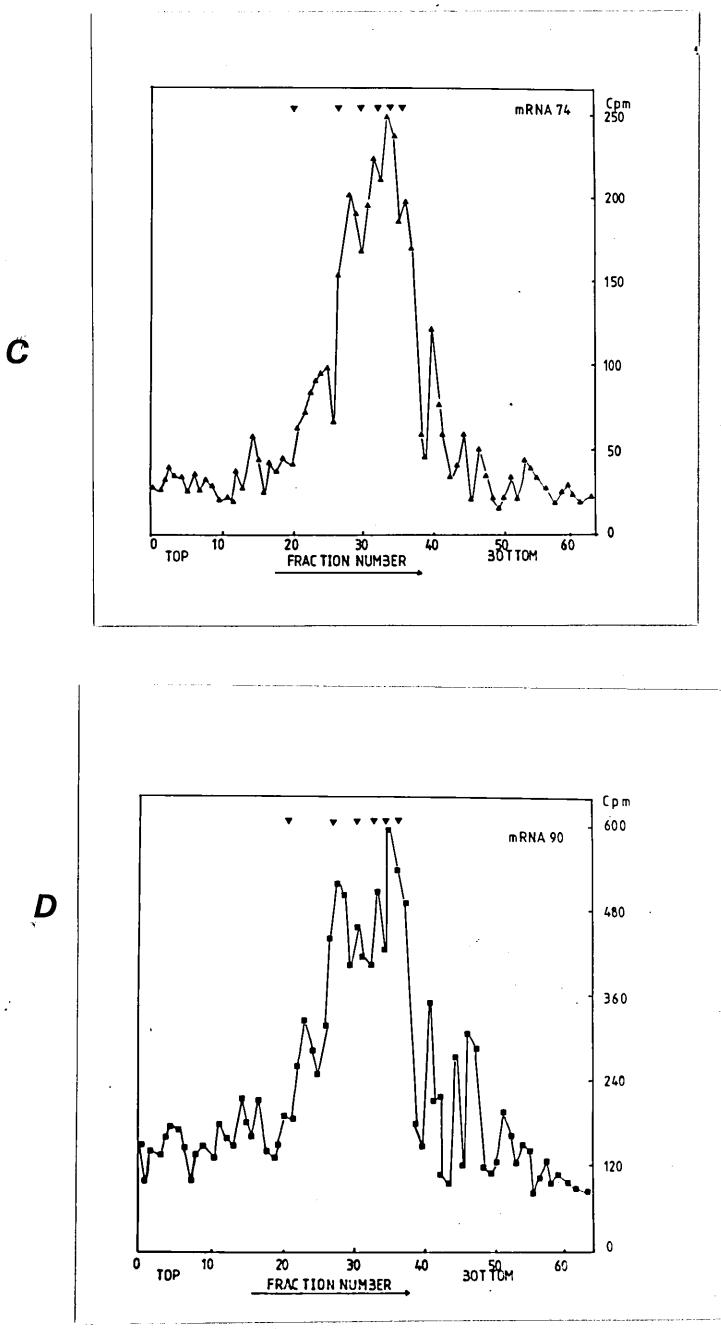


Fig.6.5. Distribution of mRNAs across polysome gradient. The radioactivity corresponding to mRNA39, mRNA100, mRNA74 and mRNA90, present in each of the dot was measured by scintillation counting. A graph was plotted of fraction number versus the cpm of each dot (A,B,C and D corresponds to mRNA39,100,74 and 90 respectively). The position of the polysomal peaks of the gradient (from which the dot blots were obtained) are indicated (▼) on the top. Direction of sedimentation is shown by the arrow.

in translation and hence in the relative distributions of specific mRNAs. However, the analysis of different mRNAs using identical dot blots from the same polysome gradients does allow valid conclusions to be drawn. Also, mRNA length will affect ribosome loading, and therefore, care must be taken to compare mRNAs of similar length. (Unfortunately, the lengths of the coding regions of these mRNAs are not known.) Hence, the polysome profiles of mRNAs 74 and 90, are of importance as these mRNAs are of similar length. The polysomal distributions of these mRNAs are similar, but their half-lives are significantly different. Therefore, no real correlation seems to exist betweenribosome loading and half-life for the mRNAs analysed in this study. This strongly suggests that ribosome loading on an mRNA does not influence its stability to a major extent and that differences in translation cannot account for the wide range of mRNA half-lives which has been observed in Saccharomyces cerevisiae.

6.3. DISCUSSION

mRNAs 90 and 74 have similar distributions on polysome gradients, but have half-lives of 7 and 85 minutes, respectively (Table 6.1). This suggests that the ribosome loading on an mRNA is not related to its half-life.

Since chemical mRNA half-life measurements were performed using hybridization techniques in this study, it was possible that the long half-lives of some mRNAs may have been due to their existence in the form of messenger ribonucleoprotein particles (mRNPs) which can be relatively resistant to

	mRNA	DISTRIBUTION OF mRNA ON POLYSOMES (PERCENTAGE OF TOTAL)				HALF-LIFE (MINUTES)	LENGTH (BASES)
		<1	1	2-4	>5		
GRADIENT 1	39	10	15	60	15	18.3 ± 1.5	380
	46	9	11	49	31	>100	550
	74	4	10	60	26	83.4 ± 9.2	1,050
	82	7	15	73	5	22.3 ± 3.1	370
	90	11	9	42	38	6.6 ± 0.6	1,100
	100	6	14	62	18	10.4 ± 1.1	500
GRADIENT 2	9	22	16	46	16	16.5 ± 1.5	440
	11	9	25	48	18	18.0 ± 2.2	700
	13	8	16	57	18	12.1 ± 1.0	740
	39	14	25	46	15	18.3 ± 1.5	380
	46	4	12	51	33	>100	550
	74	4	11	46	39	83.4 ± 9.2	1,050
	82	13	24	50	13	22.3 ± 3.1	370
	90	18	6	19	56	6.6 ± 0.6	1,100
	100	20	17	52	12	10.4 ± 1.1	500
	PYK	0	7	38	55	59.9 ± 7.8	1,600

Table 6.1. The percentage distribution of mRNAs on polysomes. The areas corresponding to pre-monosomal, monosomal and polysomal peaks on the graph were cut, weighed and their percentage to the total was calculated.

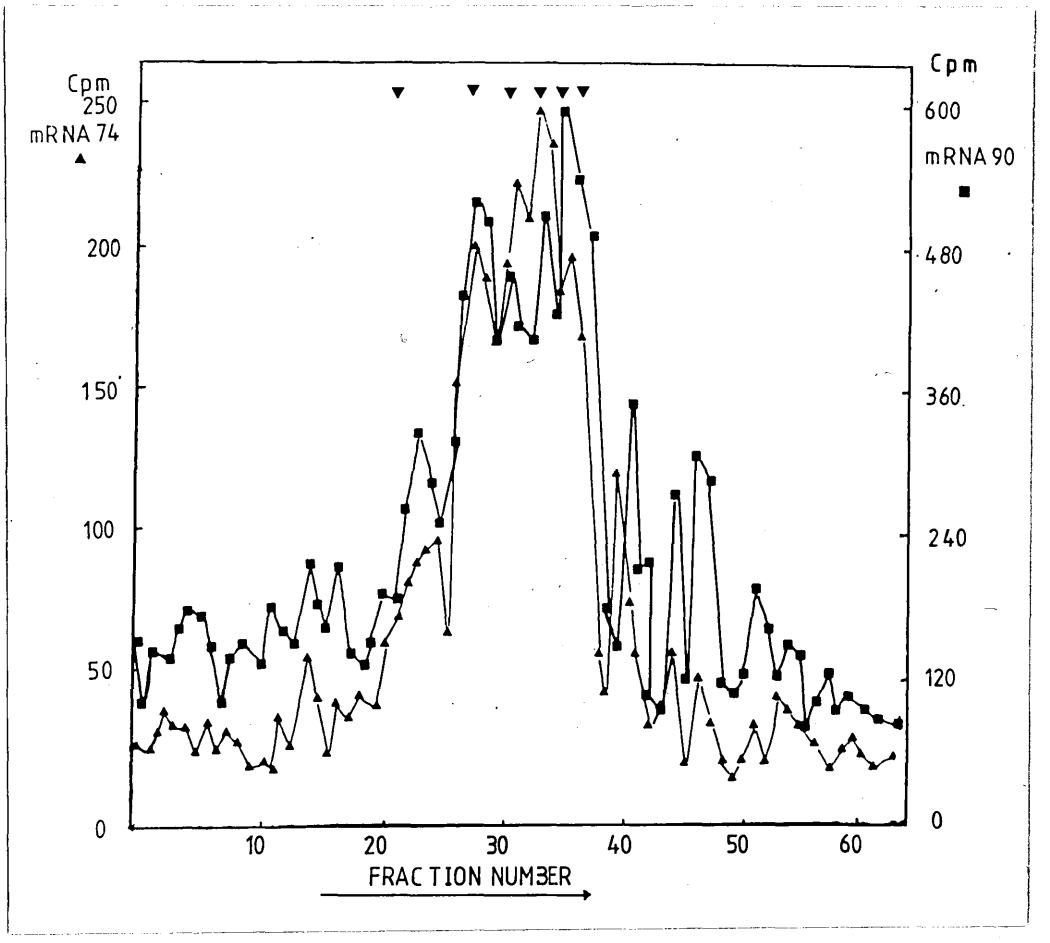


Fig.6.6. Comparison of the distribution of the mRNA74 (▲) and mRNA90 (■) across polysome gradient. Direction of the sedimentation is shown by the arrow. Position of the various polysomal peaks are indicated by (▼).

ribonucleases (Goldenberg et al., 1979; Sarkar et al., 1981). The analysis of the polysome profiles for various mRNAs showed that this is not the case. Since most of the mRNAs appear to be functional (Table 6.1) and there were no detectable degradative intermediates, it seems likely that the chemical half-lives of these mRNAs were similar to their functional half-lives.

These results are not in agreement with the earlier reports in E.coli (Morse and Yanofsky, 1969; Morse et al.,1969) where a correlation was found between ribosome loading and mRNA half-life. Since translation is closely linked to transcription in E.coli it might also follow that mRNA degradation is intimately linked with translation. In contrast, translation and transcription are separated into compartments eukaryotes and hence, mRNA degradation and translation may not be linked directly.

Superficially the results also appear to be in conflict with previous reports which suggested that mRNA translation and stability are linked (Losson and Lacroute, 1979; Pelsy and Lacroute, 1984). The authors demonstrated that premature stop codons located towards the 5'-end of the coding regions destabilized mRNAs (Losson and Lacroute, 1979; Pelsy and Lacroute, 1984). A major difference between this study and those reported previously is the analysis of functional or aberrant mRNAs. The data in this study suggest that ribosome loading is not correlated with the stabilization of specific functional mRNAs. Hence, ribosome loading does not appear to be a major factor in determining the half-life of a particular, functional

mRNA. In contrast, rendering an mRNA non-functional via the introduction of a premature stop codon appears to destabilize the mRNA. This destabilization may occur indirectly, probably by the induction of a mechanism for removal of aberrant mRNAs, rather than by a direct mechanism of non-protection through ribosome loading.

CHAPTER 7

CONCLUDING REMARKS

The stability of an mRNA is governed in part by structural features on the mRNA itself. Ideally, an investigation of the structural features which are of general importance in the determination of mRNA stability should involve the analysis of a reasonable number of mRNA species. In this study, suitable probes for analysing a number of specific mRNAs were generated by constructing a yeast cDNA library. 13 cDNA clones were isolated from the library and characterized. Southern blotting demonstrated that most of the mRNAs correspond to single copy genes with the exception of mRNA 10 which may be derived from a small gene family. Each cDNA clone yielded a single band on northern blotting and none of the cDNAs hybridized with any of the other cDNAs. Therefore, the cDNAs were suitable for use as hybridization probes to study the properties of their respective mRNAs.

A new technique has been developed to measure the chemical half-life of yeast mRNAs. In this method, a yeast culture is treated with the transcription inhibitor, 1,10-phenanthroline, and the rate of decay of specific mRNAs estimated using dot-blotting methodology. The technique is suitable for the rapid measurement of a number of mRNA half-lives under identical experimental conditions. A range of half-lives was obtained for 13 unknown mRNAs and the pyruvate kinase mRNA, from 6.6 $\pm$ 0.67 to over 100 minutes relative to the 18S rRNA control.

When mRNA lengths and half-lives were compared, two mRNA populations were apparent, both of which clearly showed an inverse relationship between mRNA length and half-life suggesting that the rate-limiting step in mRNA degradation is probably an endonucleolytic event. Since no mRNA degradation intermediates were detected by northern blot analysis the endonucleolytic digestion may be followed by rapid clearance of the cleaved mRNA molecules from cytoplasm. The existence of relatively stable and unstable populations of mRNA would explain inconsistencies in previous reports (Singer and Penman, 1973; Spradling et al., 1975; Berger and Cooper, 1975; Meyuhas and Perry, 1979). These authors suggested that there may be inverse relationships between mRNA length and half-life in a number of systems and yet each observed short unstable mRNAs and long stable mRNAs. The existence of two mRNA populations also implies that some other unknown factor(s) must strongly influence mRNA stability.

The distribution of each mRNA was analysed on sucrose density gradients of yeast polysomes to determine whether 'ribosome protection' might influence mRNA stability. If ribosomes protect an mRNA from degradation one would predict that in general mRNAs with long half-lives should carry more ribosomes than unstable mRNAs (if they are similar in length). However, no significant difference was observed in the polysome profiles of relatively stable and unstable mRNAs of similar length. These results suggest that ribosome loading is not a very significant factor in the determination of mRNA half-life in yeast.

Alternative factors which might account for the clear division of yeast mRNAs into two populations include a fundamental difference in the 5'-cap structure or the 3'-poly(A) tail, or the presence or absence of a particular sequence or secondary structure in the mRNA.

Note Added in Proof:

In this laboratory, Ian Purvis has measured the half-life of the URA3 mRNA in DBY868 (Ade2, His4) using the method outlined in Chapter 2. His estimate of 11 minutes is consistent with previous estimates of about 10.5 minutes using labelling kinetics (Losson and Lacroute, 1979) and decay kinetics (Bach et al., 1979).

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