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NORF1, a Putative Human Group I RNA Helicase That Regulates Nonsense MRNA Levels

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NORF1, A PUTATIVE HUMAN GROUP I RNA HELICASE THAT REGULATES NONSENSE mRNA LEVELS

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LIST OF ABBREVIATIONS

5'	five-prime
3'	three-prime
A	adenine
APS	ammonium persulphate
bp	base pair(s)
BPB	bromphenol blue
BSA	bovine serum albumin
C	cytosine
°C	degrees Celcius
Chisam	Chloroform:isoamyl alcohol (24:1)
Ci	Curie
CIF	cytoplasmic immunofluroescence
Cμ	constant region of μ gene
CMV	cytomegalovirus
cpm	counts per-minute
DEPC	diethyl pyrocarbonate
dFA	de-ionized formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxy nucleotides
DTT	dithiothreitol

EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
FBS	fetal bovine serum
FITC	fluroescine isothiocyanate
G	guanine
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GIT	guanidine isothiocyanate
HCl	hydrochloric acid
HEPES	N-[2-hydroxyethylpiperazine-N'-[2-ethanesulfonic acid]]
Ig	immunoglobulin
IPTG	isopropyl β-D-thiogalactoside
kb	kilobase
kDa	kilodalton
L	liter
LB	Luria broth medium
β-ME	2-mercaptoethanol
μCi	microCurie
μg	microgram
μl	microliter
ml	milliliter
mM	millimolar

M	molar
mol	moles
mAb	monoclonal antibody
MOPS	3-[N-morpholino]propanesulfonic acid
MW	molecular weight
μ	heavy chain polypeptide of IgM immunoglobulin
NaCl	sodium chloride
neo	neomycin phosphotransferase gene
nonsense mRNA	a mRNA containing a nonsense codon
nt	nucleotide
P	promoter
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBSF	phosphate buffered saline with BSA and NaN_3
PCR	polymerase chain reaction (trademark of Perkin Elmer Cetus Co.)
PEG	polyethylene glycol
PMSF	phenylmethanesulfonyl fluoride
Poly(A)	DNA polyadenylation
Prot K	proteinase K
RNA	ribonucleic acid
mRNA	messenger RNA

tRNA	transfer RNA
rRNA	ribosomal RNA
RNAse	ribonuclease
rpm	revolutions per-minute
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SSC	standard saline citrate (15 mM sodium citrate, 0.15 M sodium chloride)
SV	simianvirus
T	thymine
TAE	Tris-acetic acid-EDTA [40 mM Tris, 1% glacial acetic acid, 1 mM EDTA]
TBE	Tris-borate-EDTA [89 mM Tris, pH 7.5, 89 mM boric acid, 2 mM EDTA]
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylenediamine
Tris	Trizma base
U	unit
3'UTR	3' untranslated region
5'UTR	5' untranslated region
vol	volume(s)
X-Gal	5-bromo-4-chloro-3indolyl β-D-galactopyranoside

ABSTRACT

mRNAs containing nonsense codons encode truncated polypeptides that can interfere with the function of their corresponding full-length polypeptides. However, levels of most nonsense mRNAs are reduced in eukaryotic and prokaryotic cells when compared to that of corresponding functional mRNAs. Genes that encode proteins responsible to selectively reduce levels of nonsense mRNA have been identified only in lower eukaryotes. Here, I describe the cloning of a putative human group I RNA helicase whose amino acid sequence is 60% identical to that of UPF1, a bona fide yeast group I RNA helicase required for rapid degradation of nonsense mRNA. Besides the seven RNA helicase consensus motifs, UPF1 and its human homologue share two similar zinc finger-like domains, which are absent in other group I RNA helicases. Blocking of protein expression by antisense RNA derived from the human gene increased levels of nonsense immunoglobulin heavy chain mRNA in a mouse hybridoma line. I, thus, have identified the first mammalian protein that regulates levels of nonsense mRNA, and I have named it NORF1 (nonsense mRNA reducing factor 1).

INTRODUCTION

Nonsense codons are able to decrease the stability of mRNA (or pre-mRNA) in simple and complex eukaryotic organisms. Recently, the greatest advances to understand how nonsense mRNA levels are reduced have been made in yeast. For example, *cis*-specific nonsense mRNA targeting sequences (Peltz et al., 1993) and genes whose products are required for reducing levels of nonsense mRNA (*UPF/NMD* genes) (Leeds et al., 1991, 1993; He and Jacobson, 1995; Lee and Culbertson, 1995) have been discovered. In eukaryotic cells, it seems that the effect of a nonsense codon on mRNA depends on its position and also that levels of nonsense mRNA are reduced in the nucleus and cytoplasm (reviewed in Peltz et al., 1994). However, no mammalian nonsense mRNA reduction factor (NORF) has as yet been identified.

Using a combination of searching nucleic and protein sequence databases with the UPF1 amino acid sequence and screening complementary DNA (cDNA) libraries, I isolated a human cDNA clone that encodes a structural homologue of yeast *UPF1*, a protein that rapidly induces the degradation of nonsense mRNA. I also showed in RNA antisense experiments that the mammalian protein controls levels of nonsense immunoglobulin μ heavy chain

mRNA (nonsense μ mRNA). Therefore, I named this protein NORF1 (for nonsense mRNA reducing factor 1).

CHAPTER II

REVIEW OF RELATED LITERATURE

Nonsense Codons: Definition, Generation and Significance

Definition

Genetic information must be relayed through RNA (mRNA) before it is disseminated within a cell as a functional protein. After mRNA is transcribed from a gene, it is transported into the cytoplasm. There, ribosomes use the mRNA as a template and along with amino acid-charged t-RNAs, and translational initiation, elongation, and termination factors, synthesize a complete protein. The ribosomes translate the mRNA from the authentic start to the authentic stop signal as they move along the mRNA (a functional mRNA molecule). Through a number of processes to be described below, a mRNA can contain a premature stop signal (nonsense codon) that would end protein synthesis (Crick et al., 1961). In this case, an incomplete, truncated, polypeptide would be synthesized. Analyses of lower eukaryotic and mammalian cells containing mRNAs with nonsense codons (nonsense mRNA) have generally

found that the average (steady-state) level of nonsense mRNA is lower when compared to the same mRNA without a nonsense codon.

Generation

Nonsense mRNAs can be generated by many different processes, such as inaccurate or incomplete RNA splicing, incorrect RNA editing, mutations of DNA or RNA, or the inaccurate assembly of DNA fragments leading to nonproductive gene rearrangements. Studies of intron splicing in pre-processed mRNAs (pre-mRNA) have found that incorrect splicing can lead to the imprecise assembly of exons, exon skipping, or the failure to remove an intron altogether (intron retention) (Senapathy, 1986; Senapathy et al., 1990; Carothers et al., 1993; Stover et al., 1993). All the events can lead to a change in the translational reading frame of the mRNA and the new reading frame may contain a premature translational stop, or nonsense codon (Legrain and Rosabash, 1989; He et al., 1993).

The rearrangement of immunoglobulin (Ig) and T cell receptor gene segments as well as hypermutation of immunoglobulin genes, may also result in the production of a nonsense mRNA (Baumann et al., 1985, Lozano et al., 1993). Many mRNAs with nonsense codons can also arise from random mutations occurring within the cell's genome. Examples include single nucleotide (nt) changes (Orkin and Kazazian, 1984; Satoh et al., 1988; Jäck et al., 1989; Kadowaki et al., 1990; Hamosh et al., 1992; Longo et al., 1992; Bach

et al., 1993; Dietz et al., 1993; Gibson et al., 1993; Naylor et al., 1993; Das et al., 1994), insertions (Myerowitz and Costigan, 1988; Naylor et al., 1993; Das et al., 1994; Boles and Proia, 1995), or deletions (Krawczak and Cooper, 1991; Naylor et al., 1993; Sherrat et al., 1993, Das et al., 1994).

Significance

The presence of a nonsense codon in an mRNA usually results in a decrease in the steady state level of nonsense mRNA that is not due to decreased transcriptional rates (Cheng and Maquat, 1993; Urlaub et al., 1989; Jäck et al., 1989; Paw and Neufeld, 1988). It has been hypothesized that this process, which we call nonsense codon-mediated mRNA reduction, is used by a cell to eliminate nonsense mRNAs that could be translated into a shorter/truncated protein or a protein with a new amino acid sequence. However, if nonsense mRNA levels are not decreased, shorter proteins with the new amino acid sequence could interfere with the full-length corresponding polypeptide. For example, when a nonsense codon is present in the human β-globin gene at certain positions, levels of the nonsense mRNA are not decreased (Table 1) (Kazazian et al., 1992; Thein, 1992; Hall and Thein, 1994). Because levels of nonsense β-globin mRNA increases, a truncated β-globin encoded by the nonsense mRNA can accumulate in the cell. Patients with a heterozygous mutation in the β-globin gene produce the truncated and full length β-globin protein, but still have β-thalassemia. It has been hypothesized that the

truncated β-globin protein is interfering in a dominant-negative fashion with the function of the complete β-globin protein (Kazazian et al., 1992; Thein, 1992). Humans with this heterozygous condition are diagnosed with partial β-thalassemia, a disease manifesting itself with symptoms of anemia and bone deformation.

The most striking evidence that nonsense codon-mediated RNA degradation is important for an organism comes from experiments with the nematode *Caenorhabditis elegans* (Pulak and Anderson, 1993). In *C. elegans* a nonsense codon within one allele of the myosin heavy chain gene *unc-54* has no effect on the viability of the heterozygotic worm or on the ability of muscle cells to form a functional muscle. When the heterozygotic worm has a defect in the nonsense codon-mediated RNA degradation system, (*SMG2* mutation), the *unc-54* mRNA with the nonsense codon is not degraded and a truncated myosin heavy-chain polypeptide translated from the nonsense mRNA interferes with the assembly of muscular structure in the worm. This leads to defective muscle function so that the worms are unable to swim. Pulak and Anderson (1993) conclude that the shorter myosin heavy-chain polypeptide acts in a dominant-negative fashion and suggest that a nonsense mRNA surveillance mechanism is important to avoid the accumulation of dominant-negative or gain-of-function polypeptides.

There are also many other examples of human diseases that correlate with the presence of nonsense mRNA and in some cases with the presence of a

shorter polypeptide. For example, certain mutations in the apolipoprotein E (APO E) gene lead to the generation of a nonsense APO E mRNA. Individuals with these mutations produce a truncated APO E receptor that is not able to help remove cholesterol from the blood and suffer from elevated plasma cholesterol levels and hyperlipoproteinemia (Lohse et al., 1992; see Table 1). The table lists other examples where a nonsense mRNA is made but whether a shorter polypeptide can cause the disease is not clear.

Nonsense mRNA Reduction: Who, Where and How (Translation, Location and Models of Reduction)

Translation

How a cell is able to differentiate between a nonsense codon and a normal translational stop codon is unknown. Ribosomes, charged t-RNAs, initiation, elongation, and termination factors are all thought to be essential to recognize codon triplets and translate mRNA (reviewed in Stansfield and Tuite, 1994). Therefore, it is thought that the same translational machinery is necessary for the recognition of a nonsense codon. The inhibition of translation using the translation elongation inhibitor cycloheximide results in increased levels of nonsense mRNAs (Qian et al., 1993; Lozano et al., 1994; Menon and Neufeld, 1994; Li et al., unpublished results). These results support the hypothesis that translation is needed to reduce levels of nonsense mRNA.

Table 1. --Human Diseases Associated With a Nonsense Codon Mutation[a]

Gene	Nucleotide Change	Disease Symptoms	Truncated Protein	Nonsense mRNA Reduction	Reference
β-globin	FS > NC, NC	β-thalassemia / Cell membrane damage and destruction of red cells	Yes	No	Hall & Thein, 1994
alphaTTP	FS > NC	Vitamin E deficiency / Ataxia & Peripheral neuropathy	N.D.	N.D.	Hentati et al., 1996
BRACA1	NC	Breast and Ovarian cancer	N.D.	N.D.	Serova et al., 1996
BRACA2	NC	Breast cancer	N.D.	N.D.	Tavtigian et al., 1996
apo E2	FS > NC	Type 3 Hyperlipoproteniemia /	Yes	N.D.	Feussner et al., 1996
RB	FS > NC	Retinoblastoma / retinal tumors that can proceed to the brain	N.D.	Yes	Kato et al., 1994
SCH	NC	Schwannomas / benign tumors of the cranium and spine, Neurofibromatosis	N.D.	N.D.	Bijlsma et al., 1994
gp91-phox	FS > NC	Chronic granulomatous disease (superoxide deficiency) / recurrent life-threating infections	N.D.	N.D.	Rabbani et al., 1993
hMLH1&2	NC	Heridetiary nonpolyposis colon cancer	N.D.	N.D.	Luce et al,. 1996
ARSA	FS > NC	Metrochromatic leukodystrophy (arylsulfatase A) / defective myelin sulfatide degradation leads to demyelination and death.	N.D.	Yes	Pastor-Soler et al., 1994

Table 1. cont --Human Diseases Associated With a Nonsense Codon Mutation[a]

GP 1b alpha	FS > NC	Bernard-Soulier syndrome (absence of platelet glycoprotein Ib/IX/V complex) / giant platelets that cannot promote clotting	Yes	N.D.	Simsek et al., 1994
CDKN2	FS > NC	Putative melanoma susceptibility gene	N.D.	N.D.	Walder et al., 1995
IL-2R γ	NC	Severe combined immunodeficeincy / immunodeficiency disease (T cell def.)	N.D.	N.D.	Noguchi et al., 1993
GHRH-R	NC	Laron syndrome / Pituitary adenomas and severe growth failure	N.D.	N.D.	Hashimoto et al., 1995
WASP	FS > NC NC	Wiskott-Aldrich syndrome / X-linked immunodeficiency, thrombocytopenia and eczema	N.D.	N.D.	Wengler et al., 1995
btk	FS > NC	X-linked agammaglobulinemia / inherited immunodeficiency disease (B cell def.)	N.D.	N.D.	Duriez et al., 1994
XPAC	NC	Xeroderma pigmentosum / Skin cancer	N.D.	N.D.	Satokata et al., 1992
LAMC2	FS > NC	Herlitz's Junctional epidermolysis bullosa (laminin-5) / skin blistering at sites of pressure or trauma	N.D.	Yes	Vailly et al., 1995
p53	NC	CMML, AML, CML, Burkitt's lymphoma, Bladder cancer, endometrial carcinoma	Yes, No	No, Yes	Kawasaki et al., 1994
PHKA2	NC	X-linked liver glycogenosis type 1 / hepatomegaly, hypoglycemia, gout	N.D.	N.D.	Hendricks et al., 1995
WT1	NC	Denys-Drash syndrome / nephropathy, and Wilms tumors	N.D.	N.D.	Baird et al., 1992

Table 1. cont --Human Diseases Associated With a Nonsense Codon Mutation[a]

HEXA and HEXB	FS > NC	Tay-Sach or Sandhoff disease respectively (lysosomal β-hexosaminidase) / motor and mental deterioration, blindness	N.D.	Yes	McInnes et al., 1992
α-II β-3	FS > NC	Glanzmann's thrombasthenia / defect in platelet alpha IIb beta 3 protein	No	N.D.	Schlegel et al., 1995
subunit A	FS > NC	Subunit A coagulation factor 13 deficiency / coagulation deficiency	N.D.	Yes	Vreken et al., 1995
CD18	FS > NC	Leukocyte adherence deficiency / recurrent infections due to deficiency in surface expression of leukocyte integrin molecules	N.D.	N.D.	Back et al., 1992
CHM	FS > NS	Choroideremia / X linked progressive degeneration of the choroid and retina	N.D.	N.D.	Sankila et al., 1992
APKD1	NC	Autosomal dominant polycystic kidney disease / 1:1000 persons, massive kidneys, hematuria, hypertension, and renal failure	N.D.	No	Turco et al., 1995
ACTH-R	NC	Isolated gluocorticoid deficiency / progressive primary adrenal insufficiency	N.D.	N.D.	Tsigos et al., 1993
COL7A1	FS > NC	Hallopeau-Siemens dystrophic epidermolysis bullosa / loss of dermal adherence with abnormal anchoring fibrils	No	Yes	Hilal et al., 1993
COL10A1	FS > NC	Schmid metaphyseal dysplasias (type 10 collagen) / metaphyseal dysplasia, poor collagen assembly	Yes	N.D.	Bonaventure et al., 1995
COL4A3	FS > NC	Alport syndrome (type IV collagen)/ Deafness, hematuria, proteinuria and renal failure	N.D.	N.D.	Ding et al., 1995

Table 1. cont --Human Diseases Associated With a Nonsense Codon Mutation[a]

PBDA	FS > NC	Intermitent porphyria / skin lesions	N.D.	N.D.	Mgone et al., 1993
cytb5r	FS > NC	Cytochrome b5 reductase deficiency / syanosis or mental retardation	N.D.	N.D.	Vieira et al., 1995
FBN1	NC	Marfan syndrome (aberrant fibrillin) / connective tissue disorder affecting the skeleton, eye, and cardiovascular system	Yes	No	Dietz et al., 1994
APO E	NC	Apolipoprotein E deficiency / elevated plasma cholesterol levels, hyperlipoproteinemia	Yes	No	Lohse et al., 1992
Spectrin-β	FS > NC	Hereditary elliptocytosis /	Yes	N.D.	Wilmotte et al., 1994
OA1	FS > NC	Ocular albinism / impaired visual acuity, retinal hypopigmentation	N.D.	No	Bassi et al., 1995
ASM	FS>NC, NC	Niemann-Pick disease / neuronopathic	Yes	N.D.	Takahashi et al., 1992
SMN	FS > NC	Spinal muscular atrophy / degeneration of motor neurons of the spinal cord	N.D.	N.D.	Bussaglia et al., 1995

[a]This is a partial list of various human diseases correlating with the presence of a nonsense codon in a gene. Nucleotide changes are either mutations that generate a nonsense codon (NC) or cause a frame shift (FS) that leads to the generation of a nonsense codon. In both cases, translation is presumabely truncated and, thus, only a shorter polypeptide could be produced. N.D., not determined.

Although treatment of cells with cycloheximide seems to support the role of translation in nonsense mRNA reduction, these experiments are difficult to interpret, since the treatment itself may inhibit the production of (a) factor(s) necessary during the process of nonsense mRNA reduction. Other evidence supporting the role of translation in nonsense mRNA reduction comes from experiments using modified RNA constructs that contain a stem-loop structure that inhibits translation by virtue of its strong secondary structure (Belgrader et al., 1993). mRNAs containing these elements upstream of a nonsense codon do not undergo nonsense mRNA reduction suggesting the importance of translation in the nonsense mRNA reduction process.

Location of Nonsense mRNA Reduction

As mentioned above, the translational process is thought to be involved in nonsense mRNA reduction. Because the translational process is only known to occurs in the cytoplasm, it would be expected that the reduction of nonsense mRNA also occurs in the cytoplasm. Indeed, a number of experiments in yeast have found that nonsense mRNA reduction occurs in the cytoplasm and occurs by degradation. First, the activity of a cytoplasmic yeast 5' to 3' exonuclease (Xrn1p) correlates with the reduction of a nonsense mRNA. When a mutant strain lacks *XRN1*, cytoplasmic mRNA decay does not occur and a nonsense mRNA (the *PGK1* allele) has the same stability as a wild-type mRNA (Muhlrad and Parker, 1994). Second, the presence of a yeast factor associated with

cytoplasmic ribosomes (Upf1p) (Atkin et al., 1995) correlates with reduction of nonsense mRNA. When yeast are missing Upf1p, the levels of nonsense mRNA associated with polyribosomes increases (He et al., 1993). Both these experiments suggest that a nonsense codon in an mRNA is recognized by the translational machinery and that the reduction in the level of the message is due to degradation.

There are also experiments in mammalian cells that suggest that the reduction of nonsense mRNA occurs in the cytoplasm. Cytoplasmic nonsense mRNA reduction has been demonstrated for β-globin mRNA (Orkin and Kazazian, 1984 review) and Ig μ mRNA (Li and Jäck, unpublished observations). In these experiments, β-thalassemic patients producing β-globin nonsense mRNAs had no changes in the half-life of the nonsense pre-mRNA. But the half-life of mature nonsense mRNA was reduced to only 30 minutes when compared to the wild type β-globin mRNA half life of 16 hours (Maquat, et al., 1981; Ross and Pizarro, 1983). In the Ig μ mRNA studies, northern blot analysis was used to determine the levels of cytoplasmic μ and nonsense μ mRNA at different time points after the addition of the transcripitional inhibitors actinomycin and 5, 6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB). The result indicated that nonsense μ mRNA had a half-life of 4 hours, whereas functional μ mRNA had a half-life of 16 hours. This suggests that the degradation of the μ nonsense mRNA is occurring in the cytoplasm.

Although the evidence discussed above suggests that nonsense codons in mRNAs are recognized in the cytoplasm by the translational machinery, a number of observations suggest that the levels of nonsense mRNAs are also reduced in the nucleus of a mammalian cell. Examples include the human triosephosphate-isomerase (TPI) mRNA in both mouse and human cell lines (Cheng and Maquat, 1993), the secretory major urinary protein (MUP) mRNA in mouse cells (Belgrader and Maquat, 1994), β-globin mRNA in nonerythroid cells (Takeshita et al., 1984; Baserga and Benz, 1992), viral nonsense mRNA encoded by avian sarcoma virus (src) (Simpson and Stoltzfus, 1994) and Ig μ mRNA (Li and Jäck, unpublished observations). However, differences in nuclear levels of functional and nonsense mRNA can be explained by the presence of small contaminants of cytoplasmic mRNA that co-purified with the nuclei during the isolation procedure. To address this problem, Belgrader and coworkers examined purified nuclei by light and electron microscopy and found that the nuclei were free of any detectable cytoplasmic remnants (Belgrader et al., 1994). These observations do not, however, address the possibility that cytoplasmic nonsense mRNA molecules are attached to the outside of the nuclei and, thus, co-purify with nuclei and increase the signal of "nuclear" mRNA.

There are also observations suggesting that the presence of a nonsense codon in a pre-mRNA may also affect its splicing. The presence of a nonsense codon in an immunoglobulin kappa light-chain gene results in inefficient nuclear pre-mRNA splicing (Lozano et al., 1994). This effect on splicing is thought to

lead to the decrease in cytoplasmic levels of completely spliced kappa mRNA. Splicing experiments using κ nonsense pre-mRNA and lysates from either mouse B cells or endothelial cells suggests that inefficient splicing of κ nonsense pre-mRNA can be reproduced *in vitro* (Aoufouchi et al., 1996) but only if B cell extracts are used. This suggests that the splicing inefficiency caused by the nonsense codon can only occur in B cell splicing extracts. Unfortunately, in these *in vitro* experiments controls were done to test for the presence of possible translational machinery molecules (ribosomes and other factors) that could sterically inhibit the splicing machinery. In addition to the ability of nonsense codons to affect splicing efficiency, it has also been found that certain nonsense codons can affect exon skipping, cryptic splicing, or intron retention (Naeger et al., 1992, Dietz et al., 1993, Dietz and Kendzior, 1994, Lozano et al., 1994). Dietz has proposed that nonsense codons could be recognized within nuclear pre-mRNA by a nuclear scanner. In this model, introns are "looped-out" by splicing factors, which would align the exons as in an mRNA. A nuclear scanner could look for nonsense codons within the exons of these pre-mRNA. But the idea that a nonsense codon could influence exon skipping, cryptic splicing, or intron retention is problematic considering that nonsense codon recognition is thought to be recognized by translational machinery (in the cytoplasm). It is more likely that the nonsense codons that have these types of effects are influencing the splicing process itself by changing splice site

recognition, even if they are not located near a splicing junction. Until more is known about how the splicing machinery works, it is difficult to understand exactly how these nonsense codons are affecting the splicing process.

Models to Explain Nonsense mRNA Reduction

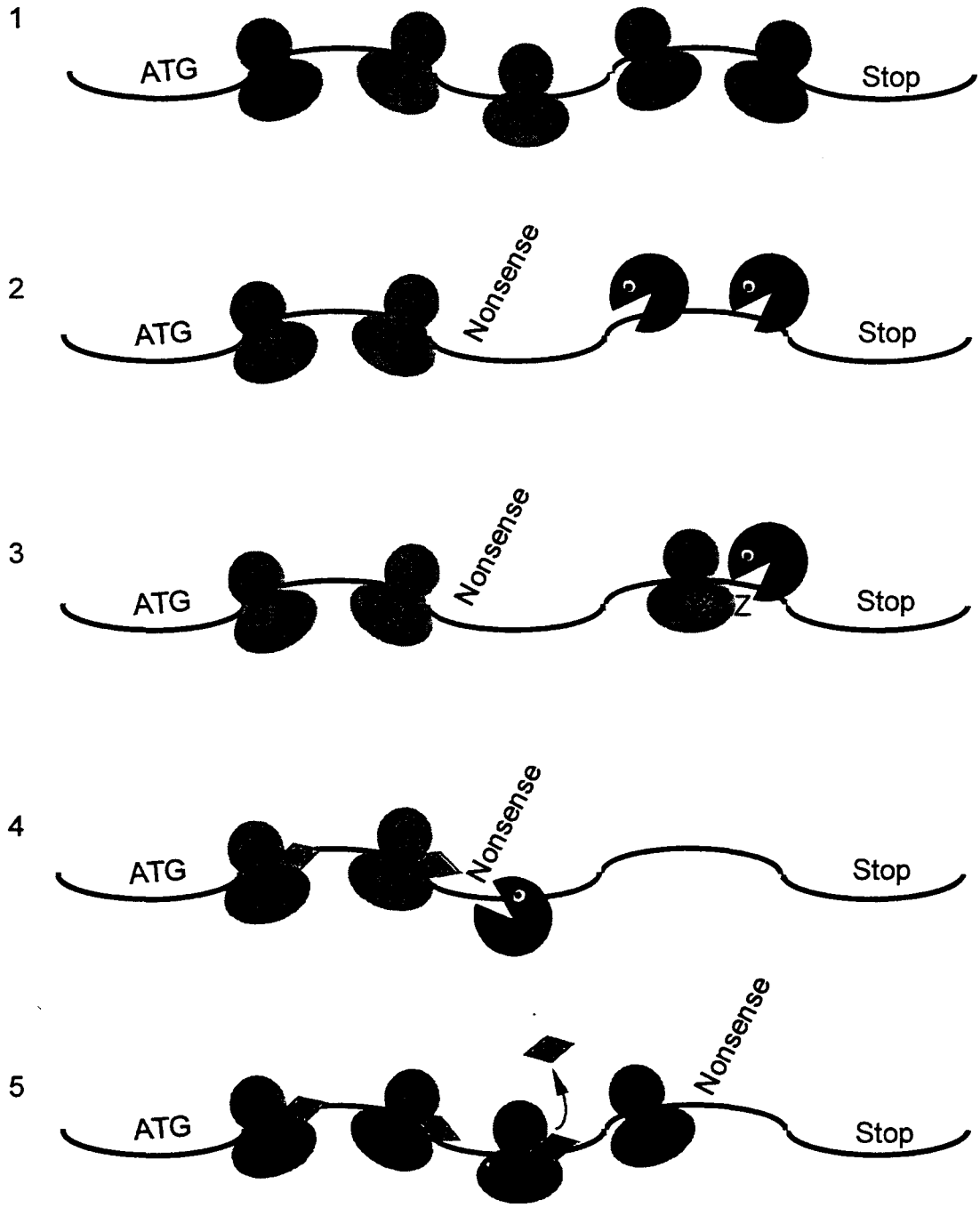
To determine how the translational machinery is able to recognize and reduce the levels of nonsense mRNA, many experiments have manipulated the location of nonsense codons in mRNAs.

Models to Explain Nonsense mRNA Reduction in Yeast

Model 1

It has been suggested that there might be a nonsense codon-recognition factor associated with the translational machinery of yeast (Figure 1.4) and after a certain percentage of the mRNA has been translated, this factor falls off gradually or by encountering a *cis*-element in the substrate mRNA (Figure 1.5) (Peltz et al., 1993). After this nonsense codon-recognition factor is removed from the translation machinery, the nonsense mRNA would not be targeted for degradation. In support of this hypothesis it has been found that the position of a nonsense codon determines whether nonsense mRNA levels are decreased when compared to levels of functional mRNA. Levels of nonsense mRNA from the yeast genes, *URA3* (Losson et al., 1983), *URA1* (Pelsy and Lacroute, 1984), *PGK1* (Peltz et al., 1993), *HIS4* (Hagan et al., 1995), and *CYC1* (Yun and

Figure 1. Models for the recognition and degradation of mRNA containing nonsense codons in yeast. 1) Normal translation of a mRNA by ribosomes 2) A nonsense mRNA that is not protected by ribosomes is a target for cytoplasmic nucleases. 3) A nonsense mRNA with a sequence downstream of the nonsense codon acts as a target for a nonsense/sequence specific decay. 4) Ribosomes containing a nonsense codon recognition factor (Small diamonds) recognize a nonsense codon and target it for decay. 5) Ribosomes containing the factor loose the factor after a certain distance or after contact with a cis mRNA element. This renders the mRNA immune to NMRD. Adapted from Peltz et al., (1993).


1
ATG
Stop
2
ATG
Nonsense
Stop
3
ATG
Nonsense
Z
Stop
4
ATG
Nonsense
Stop
5
ATG
Nonsense
Stop

Sherman, 1995), show almost complete decay when the nonsense mutation is in the 5' regions of the mRNA and normal levels when the nonsense codon is in the 3' region of the mRNA. This is called the position effect of nonsense codons on mRNA levels.

Model 2

Another hypothesis suggests that the presence of elements downstream of a nonsense codon triggers rapid mRNA decay (Figure 1.3), possibly by providing a site for 40S ribosomal translation reinitiation (Peltz et al., 1993). Experiments supporting this hypothesis found that the removal of a specific RNA sequence element 3' (an AUG rich region) from an early nonsense codon yeast *pgk1* mRNA eliminated the rapid decay of the message. The authors' main conclusion was that translational reinitiation is involved; first, because three AUG codons lie within the downstream element; second, the insertion of a stem-loop structure 5' of the downstream element inhibited the rapid mRNA decay; and inhibitors of translation that reduce the ability of ribosomes to reinitiate translation at downstream start codons also stabilize mRNA with nonsense codons without affecting the stability of a wild type transcript (Peltz and Jacobson, 1993).

Model 3

Alternatively, recent work with the yeast *CYC1* mRNA suggests a different model, the translation initiation model (Figure 2) (Yun and Sherman, 1995). This

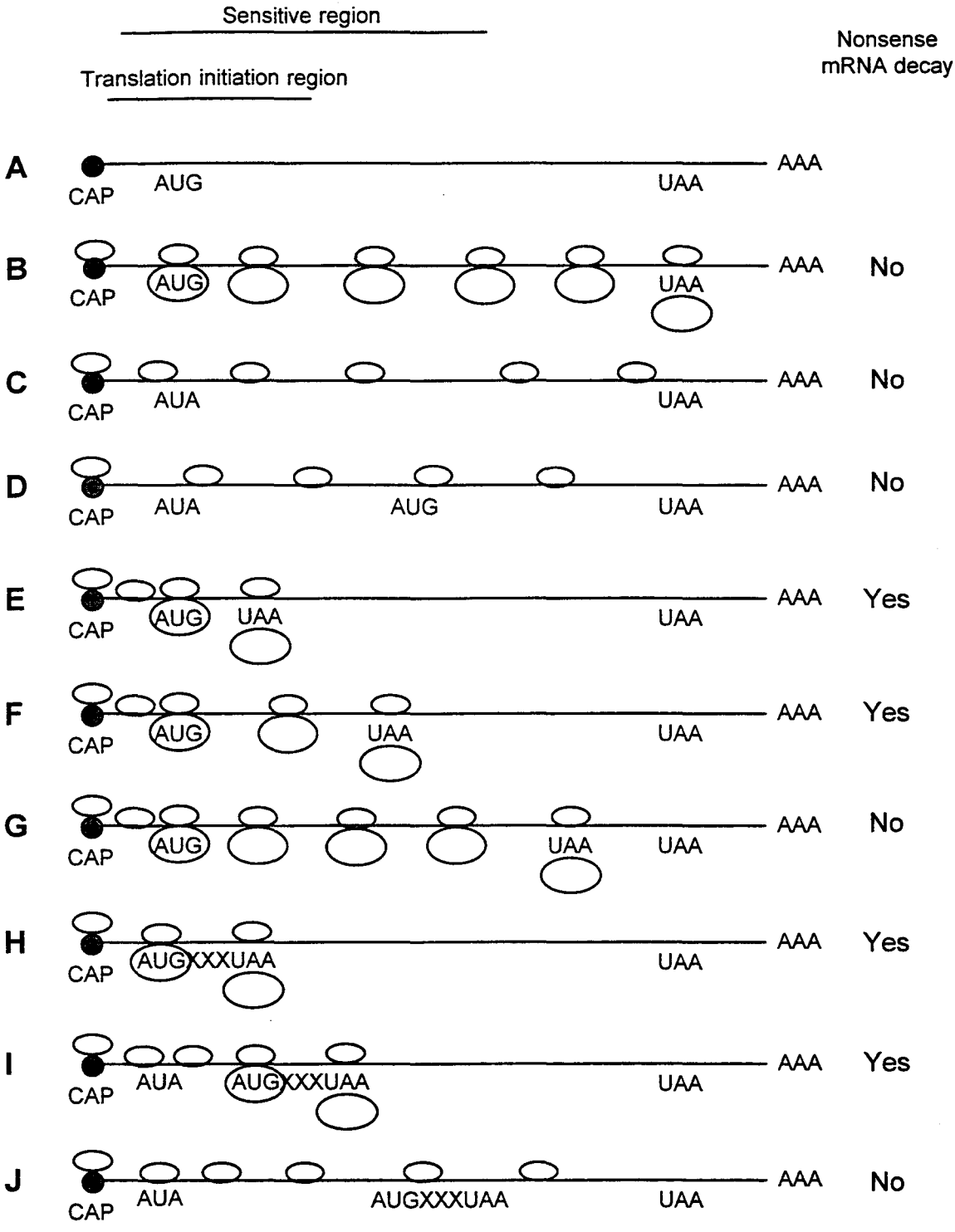


Figure 2. Schematic representation of the translational initiation model to explain effects of translation initation and termination changes on mRNA stability. The distributaion of 40S and 80S ribosomal subunits on various mRNA species are outlined. Adapted from Yun and Sherman (1995).

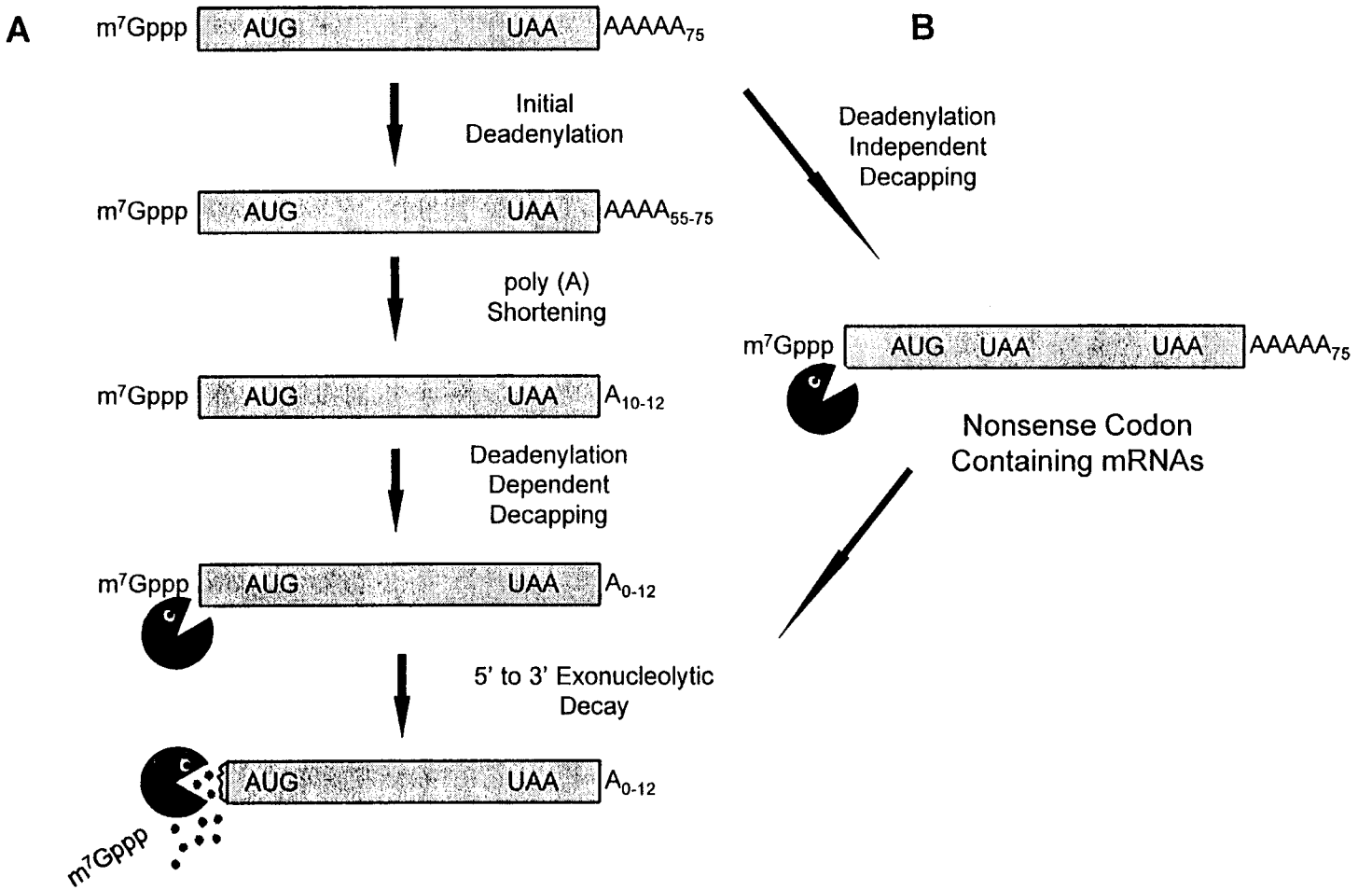
model describes a number of possible effects that *cis*-mRNA elements (including a stability element) have upon the stability of the *CYC1* mRNA. First, a *CYC1* mRNA is transported out of the nucleus (Figure 2.A) and a 40S ribosomal subunit scans the mRNA and begins translation at the bona fide AUG as a complete 80S ribosome (Figure 2.B). When the normal initiator AUG codon is changed (mutated), translation does not initiate (Figure 2.C), even when there is an AUG codon within the coding region (Figure 2.D). It may be possible that translation initiation factors are only associated with the 40S subunit for a brief period of time and are not present when a 40S subunit encounters a downstream AUG. These mRNAs are stabilized by the 40S subunit that keeps scanning down the mRNA and can cover the sensitive region. Premature termination can occur at UAA mutations at different locations (Figure 2.E, F, G) but the mRNA is only degraded if the UAA lies inside the sensitive region (Figure 2.E and F), but not when it is outside this region (Figure 2.G). This degradation occurs by exposing a sensitive element within the sensitive region. The scanning of the 40S subunit can be disrupted by inserting an ATG TAA sequence, because of the formation and quick release of 80S ribosomes (Figure 2.H, I). These nonsense codons also cause degradation of the mRNA, because they expose the sensitive element(s). However, no premature termination or destabilization occurs when the sequence ATGTAA is inserted outside of the initiation region (Figure 2.J), because the scanning 40S subunit can protect the message. The authors also suggest that the exposure of the stability element

could lead to interactions of elements with the 5' cap or the poly(A)-tail, which triggers decapping and degradation. All these models have been formulated considering results from experiments with yeast cells. The same types of elements and/or observations involving the nonsense mRNA reduction system have not yet been found in higher eukaryotic cells. Nevertheless, these results indicate that there may be special *trans*-acting factors and *cis*-mRNA elements involved in the nonsense codon mRNA recognition system in yeast but these results do not directly address how the reduction occurs.

Model 4

Experiments in yeast have addressed the question of how mRNAs containing a nonsense codon are degraded. Using temperature sensitive mRNA decay mutants, it has been found that the normal route of decay of wild-type yeast mRNAs is 1) shortening of the poly(A)-tail, 2) removal of the 5' cap of the mRNA, and 3) 5' to 3' exonuclease degradation (reviewed in Beelman and Parker, 1995) (Figure 3.A). Mulhrad and Parker (1994) demonstrated that the decay process of yeast mRNA containing a nonsense codon is similar but not identical to that of a wild-type mRNA. His lab demonstrated that the *pgk1* mRNA containing a nonsense codon does not have its poly(A)-tail shortened like a normal *PGK1* transcript but instead proceeds directly to the decapping step and then is degraded by the same 5' to 3' exonuclease that degrades wild type *PGK1* mRNAs (Figure 3.B) (Muhlrad and Parker, 1994). These results indicate that the steps in mRNA degradation are not the same for nonsense and functional mRNA

Figure 3. General mRNA decay pathway in yeast. **(A)** Pathway depicting what is thought to be the common decay pathway of a mRNA in yeast. Breifly, the process involves shortening of the poly (A) tail, deadenylation dependent decapping and subsquent 5' to 3' exonucleitic decay. **(B)** Pathway depicting the probable pathway for a nonsense codon mRNA in yeast. Breifly, the process involves deadenylation independent decapping by the NMRD mechanism and subsquent 5' to 3' exonucleitic decay similar to the decay of normal yeast mRNAs. Figure adapted from Muhlrad and Parker, (1994).


A
m7Gppp
AUG
UAA
AAAAA75
Initial
Deadenylation
m7Gppp
AUG
UAA
AAAA55-75
poly (A)
Shortening
m7Gppp
AUG
UAA
A10-12
Deadenylation
Dependent
Decapping
m7Gppp
AUG
UAA
A0-12
5' to 3' Exonucleolytic
Decay
AUG
UAA
A0-12
m7Gppp
B
Deadenylation
Independent
Decapping
m7Gppp
AUG
UAA
UAA
AAAAA75
Nonsense Codon
Containing mRNAs

and that the recognition of the nonsense codon may be the key step to activating the quick decapping of the mRNA and subsequent degradation by a common yeast exonuclease. However, it is not clear how the nonsense codon degradation system is able to recognize the nonsense codon and then activate the decapping of the mRNA.

Models to Explain Nonsense mRNA Reduction in Mammalian Cells

Model 1

As in yeast, it has been suggested that there might be a position effect of a nonsense codon on mammalian mRNA levels. Studies involving the TPI gene have shown that the levels of nonsense mRNA containing nonsense codons in upstream exons are decreased when compared to levels of functional mRNA but as the nonsense codon is relocated towards the 3' exons of the gene the levels of nonsense mRNA increase (Cheng et al., 1994). From these results Cheng and coworkers (1994) have concluded that there is also a position effect of nonsense codons upon mammalian mRNAs. These results also suggest that there may be a nonsense codon recgonition factor associated with a translated mRNA but this mammalian system is more complex than studies of intron-less yeast genes and could involve splice-site changes, possible TPI specific cis-mRNA elements, and other nuclear and/or cytoplasmic factors outlined in other models described below.

Model 2

Studies of a human nonsense β-globin mRNA in transgenic mice as well as a study done on mouse Ig μ mRNA have given some clues to the process of nonsense mRNA reduction in higher eukaryotic cells. Experiments that have looked at the β-globin mRNA and its mutant form have found that the 5' end of β-globin nonsense mRNAs have shorter, capped, 5' ends. Lim and Maquat (1992) have suggested that these nonsense mRNAs were attacked by a 5'-3' exonuclease and then capped or re-capped to confer immunity to further exonucleolytic attack. Although this model remains to be tested, evidence for the existence of a 5'-3' exonuclease has been strengthened by recent results from the lab of Brawerman. Coutts and Brawerman (1993) have identified a mouse sarcoma 5'-3' exonuclease that appears to cleave capped and uncapped mRNAs at either the first, second, or third nucleotide. Together, these results implicate a 5' to 3' exonuclease in the degradation of nonsense codon mRNAs in mammalian cells.

Li and coworkers studied the length of the poly(A)-tail in nonsense and functional μ mRNAs. In experiments using RNase H digestion followed by northern blot analysis, they found that functional μ mRNA contains a shorter poly(A)-tail than nonsense μ mRNA. This result suggests that the degradation pathway of nonsense μ mRNA does not follow normal mRNA decay which is thought to be initiated by the shortening of the poly(A)-tail (reviewed in Ross, 1995). It is possible that the degradation of nonsense μ mRNA follows a

pathway similar or identical to that of yeast *pgk1* nonsense mRNA, in which the nonsense mRNA does not have its poly(A)-tail shortened before quickly decapping and degradation by a 5' to 3' exonuclease. Experiments have also been performed to determine if other steps in the degradation of higher eukaryotic nonsense mRNAs follow a pathway similar to that of yeast.

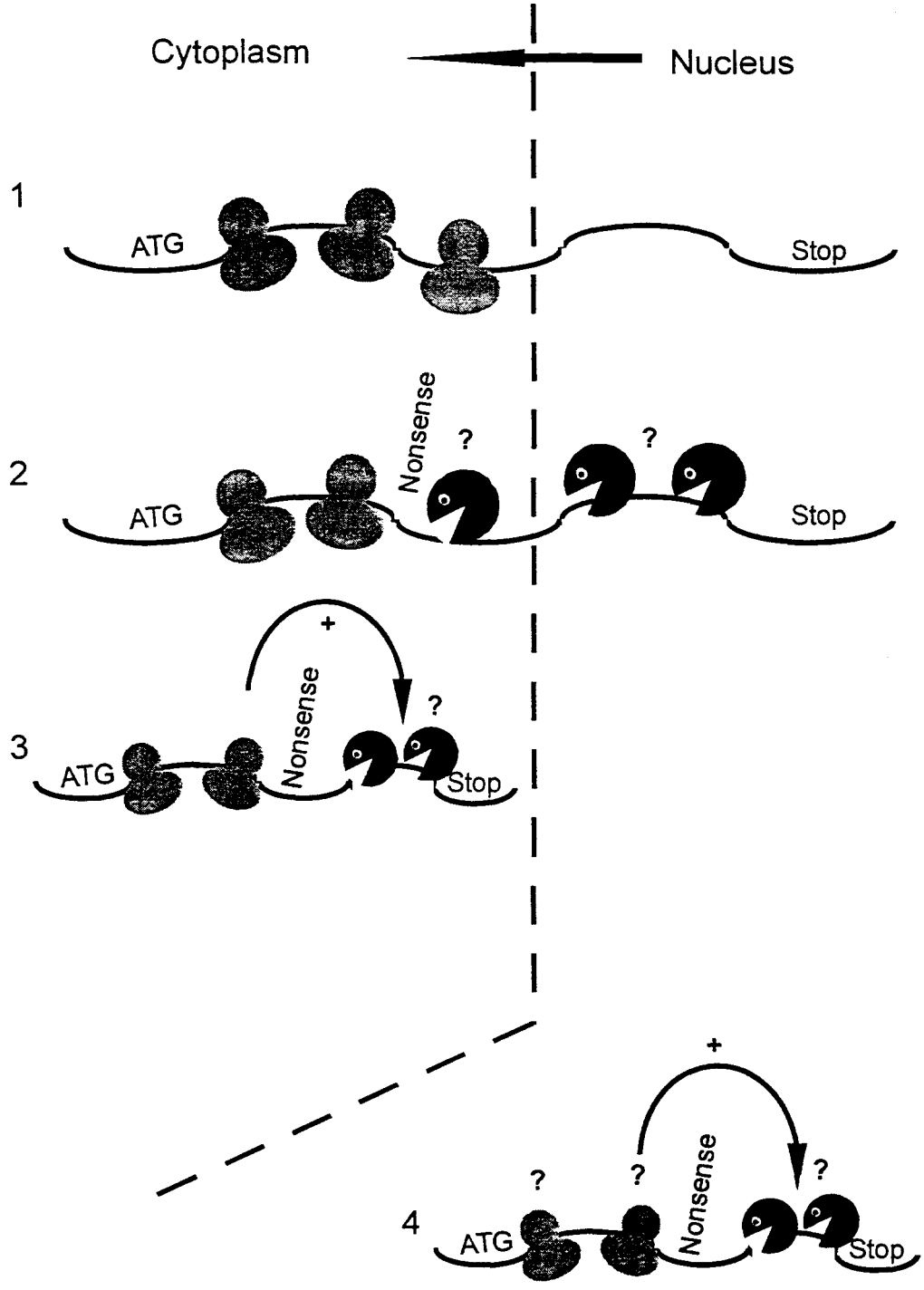
Model 3

To explain how a nonsense codon can trigger the reduction of nuclear mRNA, Chasin and coworkers have proposed the nuclear co-translational export model shown in Figure 4.2. This model explains how nonsense codons in a mRNA can be recognized by the translational machinery in the cytoplasm and still result in a nuclear decrease in the nonsense mRNA level (Urlaub et al., 1989). In this model, ribosomes encounter nascent, not completely spliced RNA at the nuclear pore and begin the translation process (Figure 4.1). When a nonsense codon is encountered, the ribosomes activate the nonsense mRNA reduction pathway either at the surface or inside the nucleus (Figure 4.2). This could lead to the degradation of the mRNA or prevent further splicing, both of which would account for a drastic reduction in cytoplasmic nonsense mRNA levels.

Model 4

An additional model of nuclear degradation of nonsense mRNA has been

Figure 4. Models for the recognition and degradation of mRNA containing nonsense codons. 1) Export of a normal mRNA using the translational-translocation model. 2) Degradation of a nonsense mRNA during translational-translocation by putative nucleases either outside or inside the nucleus. 3) Cytoplasmic degradation of a nonsense mRNA. 4) Nuclear scanning model where nonsense codons are recognized in the exons of an mRNA which targets the mRNA for degradation.


Cytoplasm
Nucleus
1
ATG
Stop
2
ATG
Nonsense
?
?
Stop
3
ATG
+
Nonsense
?
Stop
4
ATG
?
?
+
Nonsense
?
Stop

proposed by Chasin and coworkers. In this model, which was termed the nuclear scanning model, a hypothesized nuclear scanner identifies a nonsense mRNA and targets it for degradation (Urlaub et al., 1989) (Figure 4.4). Because the only known complex that can identify codons in a mRNA is tRNA, cytoplasmic ribosomes and there associated factors, it is difficult to imagine how the scanning occurs in the nucleus without a translation-like process. Recently, this scanning model has been redefined to only effect completely spliced mRNA (Zhang and Maquat, 1996). Zhang and Maquat have used a site-directed mutagenesis approach to generate a nonsense codon that spans two exons (is separated by an intron) in the triosephosphate isomerase (TPI) gene. In other words, the nonsense codon is generated by splicing two exons together and is, thus, only found in spliced mRNA. Although RT-PCR was used to determine differences in nuclear TPI RNA levels, which leads to problems quantitating exact differences in RNA levels, the evidence suggests that nuclear nonsense mRNA reduction occurs only on the completely spliced TPI mRNA and not on the TPI pre-mRNAs (Zhang and Maquat, 1996). These results suggest that if nuclear scanning occurs it would only affect completely spliced nonsense mRNAs and not nonsense pre-mRNAs. This hypothesis circumvents one of the difficulties of the nuclear scanning model which was to explain why pre-mRNAs that contain a nonsense codon within an intron are not targeted for nonsense mRNA reduction. These results, however, do not prove the existence of nuclear nonsense mRNA reduction. Once experiments are designed and carried out to

directly test the nuclear co-translational translocation and nuclear scanning models, these results may become clearer.

Genes Involved in Nonsense mRNA Reduction

Previously, researchers were only able to study the phenomenon of nonsense mRNA reduction, but recently through the use of genetic systems, researchers working with *S. cerevisiae* and *C. elegans* have identified a series of genes that are directly involved in the nonsense mRNA reduction process. In *S. cerevisiae* they are named *UPF* genes (up-frameshift, once thought to be frameshifting enhancers) and in *C. elegans* they are called *SMG* (suppressor of morphogenesis mutations gene) genes. *UPF1, 2, 3* and *SMG 2, 3, 5*, and *7* have been cloned. In any of the *UPF* mutants there is an increased steady state level of mRNAs with nonsense codons and unspliced pre-mRNAs that contain a nonsense codon within an intron (He et al., 1993). The best characterized of these genes, and the only one that will be discussed in detail is the yeast *UPF1* gene. By using genetic and molecular analysis, *UPF1* was shown to be required in the nonsense mRNA reduction pathway (Peltz et al., 1990, 1993). The levels of nonsense mRNA return to that of the corresponding wild-type mRNA, indicating that the *UPF1* gene is involved in the nonsense mRNA decay process but not in general mRNA decay.

The *UPF1* gene encodes a 109-kDa protein (Figure 5). The UPF1 protein (Upf1p) contains a number of motifs with interesting properties. The most

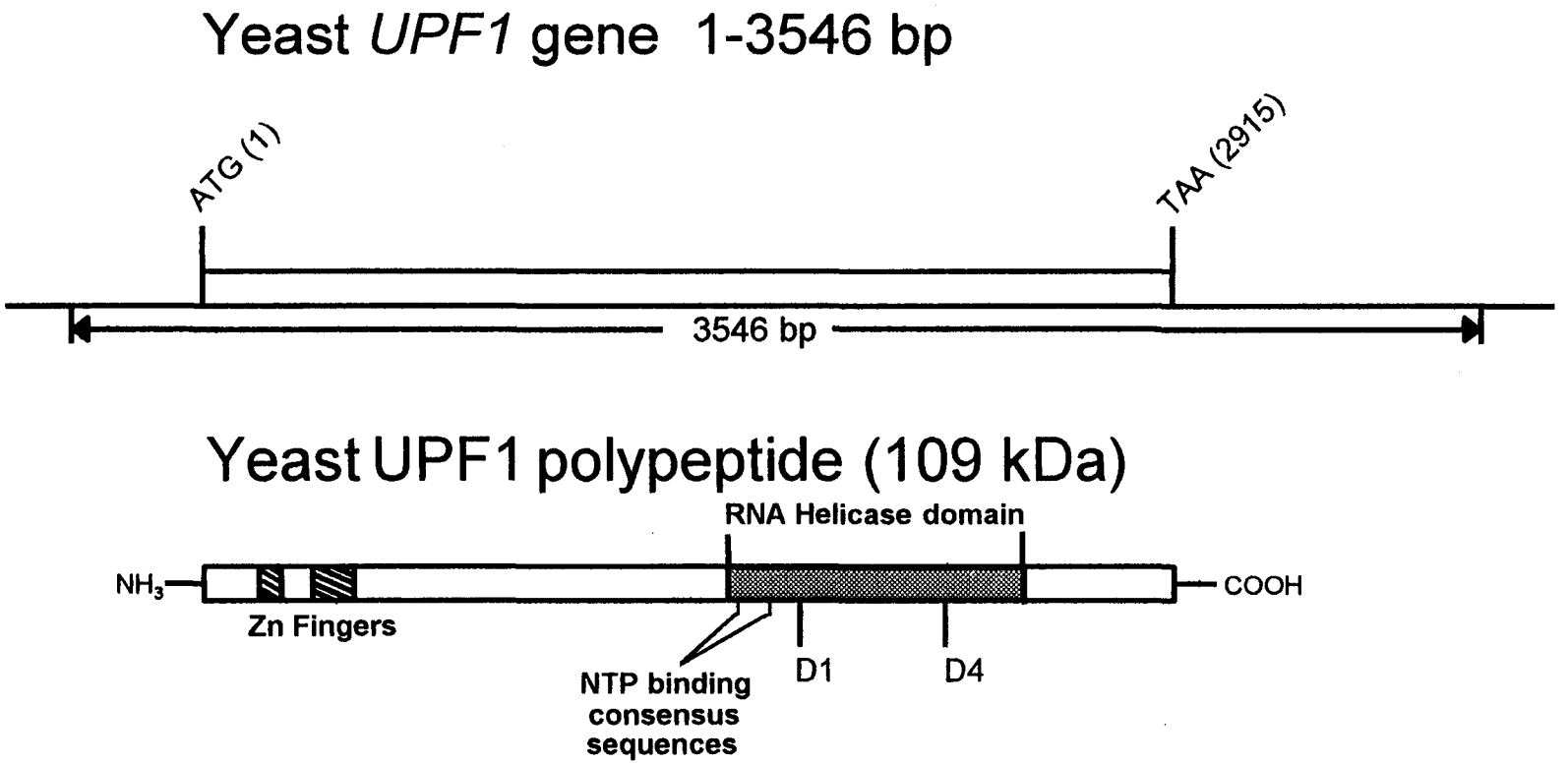


Figure 5. Schematic representation of the *S. cerevesiae UPF1* gene and gene product. UPF1-D1 and UPF1-D4 are the locations of two missense mutations that lead to the production of a dominant-negative UPF1 gene product.

thorough sequence analysis of the putative Upf1p has been performed by Altamura and coworkers and is described here in detail (Altamura et al., 1992). Within the *UPF1* ORF (from the N- to C- direction) there is a highly acidic region, a Zn^{+2} finger motif, a NTP binding domain and a type 1 (superfamily 1) RNA helicase domain (Leeds et al., 1992; Altamura et al., 1992). It has been suggested that the acidic regions of DNA-binding transcriptional factors are required for their ability to act as transcriptional activators (reviewed in Struhl, 1989). Upf1p also contains conserved cysteine residues found in a number of zinc-finger DNA and RNA binding proteins (Burd and Dreyfuss, 1994). It is thought that these cysteine residues form structures that allow interaction with helices of nucleic acids. It is possible that this motif in Upf1p could interact with ribosomal RNA (either double or single stranded) or with a nonsense mRNA. The elongation factor eIF-2β also has a zinc-finger motif. When this motif is altered, the factor can no longer help the ribosome recognize the translational AUG start codon. This suggests that a zinc-finger motif could help recognition of a codon. It is therefore possible that the zinc-finger motif present in Upf1p could be directly involved in the recognition of a stop codon. Upf1p also contains two NTPase consensus motifs (Walker et al., 1982) that are the first and second motifs (motif I and II) of the RNA helicase region of RNA helicases of the superfamily I (Koonin, 1992). Many superfamily I putative RNA helicase genes such as *SEN1* (DeMarini et al., 1992) and MOV-10 (Mooslehner et al., 1991) all contain a NTP binding domain and are thought to have NTP dependent helicase

activity. It has been proposed that the RNA helicase domain of Upf1p could facilitate the degradation of a nonsense mRNA by unwinding secondary structure downstream of a nonsense codon thereby making the RNA accessible to nucleases (Peltz et al., 1993).

Recently the functions of a number of these motifs have been confirmed (Czaplinski et al., 1995). Purified, epitope-tagged Upf1p can bind to double stranded DNA or RNA. Upf1p can be released from this substrate by the addition of ATP and in doing so, it unwinds the dsDNA or dsRNA; presumably by using its RNA helicase domain. Leeds and coworkers (1992) have shown that certain mutations in the RNA helicase domain of the *UPF1* gene confer a dominant-negative effect upon cells transformed with the mutant *upf1* (Leeds et al., 1992). These cells containing the wild-type *UPF1* and the RNA helicase mutant *upf1* act as if they are *upf1*-null. These data support the hypothesis that Upf1p may be acting as a dimer and that the RNA helicase domain is critical for the function of Upf1p.

Experiments performed by Czaplinski and Peltz suggest an even more complex picture of Upf1p function (personal communication). An *upf1* mutant used in this study changed its growth on plates without leucine depending on the levels of nonsense *leu2-1* mRNA (e.g. yeast that have a wild type *UPF1* gene and a *leu2-1* nonsense mutation do not grow in the absence of leucine, whereas yeast that have a *upf1* null allele and a *leu2-1* nonsense allele grow in the absence of leucine. In other words, the phenotype of a yeast cell with the *leu2-1*

allele and an *upf1* mutation changes from Leu$^-$ to Leu$^+$ because, in the absence of Upf1p, nonsense *leu2-1* mRNA is more stable and translational readthrough generates enough functional Leu2p to grow in the absence of leucine). Recent unpublished observations by Czaplinski and Peltz using various *upf1* yeast challenge this conclusion and shed some light on how Upf1p may be actually working. They have created a number of different *upf1* mutants in the putative zinc-finger and RNA helicase regions of the *UPF1* gene and tested the ability of the mutated Upf1p to complement the function of wild-type *UPF1 in vivo*. Their results are shown in Table 2.

Table 2. --Mutational analysis of *UPF1* and its effects on a nonsense mRNA *in vivo*[a]

Mutation Group	Phenotype		Specific Mutations Made in *UPF1*
	Nonsense *leu2-1* mRNA levels	Leu phenotype	
A	Up	+	H94→R / H98→R / C122→S / C125→S / K436 to P, E, D, Q, or A
B	Down	-	C72→S / H110→R / RR793→KK
C	Up	-	DE572→AA / RR793→AA / TR800→AA
D	Down	+	C65→S / C84→S / C148→S

[a]Phenotype indicates either high (up) or low (down) levels of the *leu2-1* mRNA containing a nonsense codon while a + indicates growth, or - indicates lack of growth on drop-out medium. Specific mutations are indicated by their IUPC amino acid code. The wild-type residue and location of changes are indicated on the left before the arrow. The arrow (→) indicates the type of change that has been made.

These results demonstrate that Upf1p is not only necessary for the decay of nonsense mRNAs, (Group A and C), but it may also be involved, either directly or indirectly, in modulating the recognition of the nonsense codon (Group A and D).

Recently, *UPF2/NMD2*, which is called *NMD2* (for nonsense-mediated mRNA decay gene 2) has been cloned with the yeast two-hybrid system using the Upf1p as "bait" (Cui et al., 1995; He and Jacobson, 1995). The way in which Upf2p was found implies that it interacts with Upf1p. Additionally, dominant-negative fragments of Upf2p expressed in the cytoplasm of yeast are able to interfere with the nonsense mRNA reduction pathway. However, the same Upf2p fragment located to the nucleus does not prevent nonsense mRNA degradation. This not only indicates that Upf2p interacts with Upf1p and could be a component that recognizes a nonsense codon, but it also shows that the yeast nonsense mRNA reduction can occur in the cytoplasm. The identification of the yeast *UPF3* gene could also help researchers that study nuclear nonsense mRNA reduction understand how this may occur. Upf3p contains a bi-partite yeast nuclear localization signal and, therefore, is thought to be present in the nucleus. The mammalian homologue of this gene (if it exists) would become a good candidate for a factor involved in nuclear nonsense mRNA reduction.

As mentioned above, Parker's studies of yeast genes involved in general mRNA decay have indicated that the process involves 1) shortening of the poly(A)-tail, 2) decapping of the message and finally 3) 5' to 3' exonuclease

activity that degrades the mRNA. His studies of a mRNA containing a nonsense codon (*pgk1*) also found that the decay proceeds directly to the decapping step and then follows the normal decay pathway. His lab also studied whether the *UPF1* gene played a role in the decapping process. In experiments using a yeast strain containing an *UPF1* mutation, a mutation in the 5' to 3' exonuclease, and an mRNA with a nonsense codon, they found that the nonsense mRNA contained a shortened poly(A)-tail and eventually was decapped at a rate similar to a wild type mRNA. From this observation the authors concluded that *UPF1* is involved directly or indirectly in the quick decapping of the nonsense mRNA.

The intracellular location of Upf1p is consistent with it being involved in nonsense codon recognition and/or decay. Upf1p is predominantly found in the cytoplasm and has been shown to be associated with polysomes (Atkin et al., 1995), and in this way, it could be involved in the recognition of a nonsense codon. Yeast lacking Upf1p also have an increase in pre-mRNAs that contain a nonsense codon within an intron (He et al., 1993). Interestingly, not only is the pre-mRNA level increased in *upf1* mutant yeast, but these pre-mRNAs are also associated with ribosomes in the cytoplasm. This indicates that nonsense mRNA reduction probably needs the translation process as well as the Upf1p.

Similarities of Nonsense mRNA Reduction Between The Yeast and Higher Eukaryotic Systems

The model systems for nonsense mRNA reduction in yeast and higher eukaryotic cells contain a number of similarities. First, translation is required for nonsense mRNA reduction. Compounds and RNA structures (cycloheximide and stem-loops, respectively), both of which inhibit ribosomal elongation can prevent nonsense mRNA reduction. Second, it appears that the poly(A)-tail of both yeast and mammalian nonsense mRNA (e.g. *pgk1* and mouse μ) is not shortened before activation of nonsense mRNA reduction. This suggests that after the recognition of a nonsense codon (by a still unknown mechanism), the nonsense mRNA in yeast and mammalian cells might be degraded in a similar fashion. Third, not all nonsense codons can target an mRNA for nonsense mRNA reduction. The position of a nonsense codon within yeast or mammalian RNA determines whether a specific mRNA or pre-mRNA is targeted for nonsense mRNA reduction.

Summary

Significant effort has been put forward to study the effects of a nonsense codon on the stability of the afflicted mRNA (or pre-mRNA). Recently, the greatest advances have been made in the yeast *S. cerevisiae*. These systems have discovered possible *cis*-specific nonsense mRNA targeting elements, genes specifically involved in the nonsense mRNA reduction system (*UPF/NMD* genes) and insights into the decay of nonsense mRNA after the nonsense codon has been recognized. Research of higher eukaryotic systems is still focused at

nonsense mRNA reduction phenomenon such as nuclear v.s. cytoplasmic nonsense mRNA reduction, the effects of a nonsense codon on pre-mRNA stability and splicing, and the position effect of nonsense codons. Until the system itself is identified and studied biochemically, the process of nonsense mRNA reduction will remain an enigma. In order to understand the process of nonsense mRNA reduction in higher eukaryotic cells work needs to be done to identify the higher eukaryotic homologues of yeast nonsense mRNA reducing factors.

The Hypothesis

Nonsense codons found within mRNA frequently lead to a decreased steady state level of nonsense mRNA within an eukaryotic cell. This effect is found in simple (*S. cerevisiae*) and complex eukaryotes (vertebrate). Genes required for nonsense codon-mediated mRNA decay have been identified in *S. cerevisiae* and *C. elegans* through genetic screens. However, no mammalian nonsense mRNA reducing protein has yet been identified. Because of the general similarities between the nonsense mRNA reduction process in yeast and higher eukaryotes, and the observation that a large number of yeast genes are conserved in higher eukaryotic organisms (Tugendreich et al., 1994), I propose that nonsense mRNA reducing factors similar to those found in the yeast *S. cerevisiae* are present in mammalian cells and control levels of nonsense mRNAs.

CHAPTER III

MATERIALS AND METHODS

Chemicals and Reagents

General Chemicals and Reagents

Ampicillin (Na Salt) and kanamycin from either Sigma Chemical (St. Louis, MO) or Boehringer Mannheim (Indianoplis, IN). L-canavanine from Sigma Chemicals; AG^{R}501-X8(D) (Mix bead resin), BIS-acrylamide, gelatin, and Tween 20 from BioRad (Richmond, VA). Yeast extract, Bacto-agar, Yeast extract w/o amino acids are from Difco (Detroit, MI). Agarose (ultra-pure), Geneticin (G-418 Sulfate), cesium chloride (ultra-pure), di-thiothreiotol (DTT), LB-media base, Penicillin-Streptomycin (500 U/ml), L-Glutamine (200 mM), Glycerol (ultra pure), and RPMI 1640 powder from Gibco-BRL Life Technologies, Inc. (Gaithersburg, MD); dextran sulfate from Pharmacia (Uppsala, Sweden); fetal calf serum (FCS) from HyClone (Logan, UT); universal autoradiography enhancer chemicals (Part A and B) from DuPont NEN (Boston, MA); Triton X-100 from Serva (Heidelberg, Germany); ethyl alcohol (EtOH, 200 proof) from

paper Co. (Shelbyville, KY); Non-fat dehydrated milk from Carnation Food Products, Nestle Food Co. (Glendale, CA); scintillation liquid (Econo-Safe) from RPI (Mount Prospect, IL). All solutions were prepared in deionized water (dH_2O) filtered by a Millipore Milli-Q water filtering system (Bedford, MA).

Radiochemicals

[α-^{32}P] dCTP (Cat# PB10205)	Amersham, Arlington Heights, IL
Redivue [α-^{32}P] dCTP (Cat# AA0005)	Amersham, Arlington Heights, IL
[α-^{35}S] dATP (Cat# SJ1304)	Amersham, Arlington Heights, IL
Trans ^{35}S-label (Cat# 51006)	ICN Biomedicals, Irvine, CA
[γ-^{32}P] dATP (Cat# 35020)	ICN Biomedicals, Irvine, CA

Kits

DNA 5'-end labeling kit (Cat#702757)	Boehringer Mannheim, Indianapolis, IN
Enhanced Chemiluminescnce Kit (Cat#1500694)	Boehringer Mannheim, Indianapolis, IN
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/Diaminodipropylamine Immobilization kit (Cat#44899)	Pierce Co., Rockford, IL
Antigen Conjugation kit (Cat#77107)	Pierce Co., Rockford, IL
Nick-translation kit (Cat#18160-010)	Gibco BRL, Gaithersburg, MD

pGEM-T vector system I (Cat#A3600)	Promega, Madison, WI
Sequenase kit (2.0) (Cat#US70770)	United States Biochemical, Cleveland, OH
TNT *in vitro* translation kit (Cat#L4610)	Promega, Madison, WI
Wizard mini-prep kit (Cat#A7100)	Promega, Madison, WI
Qiagen plasmid isolation DNA kits (Cat#12143)	Qiagen, Chatsworth, CA
Qiagen total RNA kit (Cat#74104)	Qiagen, Chatsworth, CA

Antibodies

Antibodies Used for Immunofluroescence Analysis

Rabbit anti-V_H81X	Hartwell and Jäck, unpublished; Loyola University, Maywood, IL
FITC-labled goat anti-mouse μ (Cat#1021-01)	Fisher Biotech, Pittsburgh, PA
FITC-labled goat anti-rabbit IgG (Cat#9817SA)	Gibco-BRL, Gaithersburg, MD

Antibodies Used for Western Blot Analysis

Rabbit anti-UPF1 peptide antiserum	Allan Jacobson, University of Massachusetts Medical School, Worcester, MA
Horseradish peroxidase conjugated (HRP) affinity-purified goat anti-rabbit IgG (Cat#170-6515)	BioRad, Irvine, CA
Affinity-purified goat anti-MOPC104E (mouse μ)	Bornemann et al., 1995
HRP conjugated rabbit anti-Goat IgG	Fisher Biotech, Pittsburgh, PA
Affinity purified rabbit anti-NORF1/pep1	This work
Affinity purified rabbit anti-NORF1/pep2	This work

Molecular Weight Standards

1Kb DNA ladder	Gibco BRL, Gaithersburg, MD
λ DNA/*Hind*III fragments	Gibco BRL, Gaithersburg, MD
Rainbow protein marker	Amersham, Buckinghamshire, England
Bacteriophage ΦX174 DNA	Gibco BRL, Gaithersburg, MD

Enzymes

Restriction endonucleases were obtained form Boehringer Mannheim Biochemicals (Indianapolis, IN), Gibco BRL Life Technologies, Inc. (Gaithersburg, MD) and New England BioLabs, Inc. (Beverly, MA); AmpliTaq DNA polymerase from Perkin Elmer Cetus (Norwalk, CT) and Taq polymerase and Superscript reverse transcriptase from Gibco-BRL Life Technologies, Inc. T4 DNA ligase and Klenow fragment from either Pharmacia (Piscataway, NJ) or Promega (Madison, WI); RNasin and DNase I, from Promega (Madison, WI); RNase H from Pharmacia (Piscataway, NJ); RNase A (bovine pancreas) and lysozyme (chicken egg white) from Sigma Chemical Company (St. Louis, MO); DNA Sequenase (polymerase) version 2.0 from United States Biochemical (USB, Cleveland, OH); and Proteinase K from Boehringer Mannheim (Indianapolis, IN).

Oligonucleotides

Standard oligonucleotides were purchased from Promega Corp. and United States Biochemical or were synthesized by Bio-Synthesis (Lewisville, TX), Chander Raman, Gene Napolitano (Terrapin Technologies, South San Francisco, CA), or by the Macromolecular Core Facility at Loyola University (Maywood, IL).

LAC primer: 17 mer 5'-TGTGGAATTGTGAGCGG-3'

(Elledge et al., 1991), From Bio-Synthesis

GAL primer: 17 mer 5'-ACTTTAACGTCAAGGAG-3'

(Elledge et al., 1991), From Bio-Synthesis

GAL-UP.Fow: 18 mer; 5'-TTCGGTTTGTATTACTTC-3'

From Bio-Synthesis

SP6 primer: 19 mer; 5'-GATTTAGGTGACACTATAG-3'

From Promega

T7 primer: 20 mer; 5'-TAATACGACTCACTATAGGG-3'

From Promega

M13 primer (-40, forward): 17 mer; 5'-GTTTTCCCAGTCACGAC-3'

From USB

M13 primer, backwards: 16 mer; 5'-TTCACACAGGAAACAG-3'

From USB

TRAPα primer #1: 14 mer; 5'-CACTAATTCCCATA-3'

From Loyola Core Facility

TRAPα primer #3: 14 mer; 5'-CTGTTTGATTGAAG-3'

From Bio-Synthesis

TRAPα primer #4: 16 mer; 5'-CTCCCCCGCTTGCTTC-3'

From Bio-Synthesis

TRAPα primer #5: 15 mer; 5'-GTTAAGTCCCAAGCTG-3'

From Gene Napolitano

TRAPα primer #6: 13 mer; 5'-GCAAGATGAAGATG-3'

From Gene Napolitano

1RGF.hyb primer: 19 mer; 5'-CCTCAGGTTCTATTATGTC-3'

From Loyola Core Facility

2RGF.hyb primer: 16 mer; 5'-GGCATGTTTTCAGTGC-3'

From Loyola Core Facility

UPF1-D1.Forward primer: 15 mer; 5'-GGAAAACAGAAGCAG-3'

From Bio-Synthesis

UPF1-D1.Backward primer: 15 mer; 5'-CGGGTTAGACCCACG-3'

From Bio-Synthesis

2.0H35hom.forward primer: 26 mer;

5'-GATGTTGACATGTGTTGGTGCTGGTG-3'

From Loyola Core Facility

2.0H35hom.backward primer: 26 mer;

5'-CACCAGCACCAACACATGTCAACATC-3'

From Loyola Core Facility

SAM#3.Fow: 20 mer; 5'-GCTGATCCTTGTAGGCGACC-3'

From Loyola Core Facility

SAM#4.Bak: 20 mer; 5'-GGTCGCCTACAAGGATCAGC-3'

From Loyola Core Facility

SAM#5.Bak: 18 mer; 5'-CCTGGGACAGCTCCGGCT-3'

From Loyola Core Facility

SAM#6.Fow; 18 mer; 5'-GGACAGCTCGACGCGCAG-3'

From Loyola Core Facility

SAM#7.bak: 18 mer; 5'-CTGCGCGTCGAGCTGTCC-3'

From Bio-Synthesis

SAM#8.bak : 20 mer; 5'-GCGTCTGCGTCTGGCTAGGA-3'

From Bio-Synthesis

SAM#9.bak: 19 mer; 5'-CAAGCAGCTCGGCCTCCTC-3'

From Bio-Synthesis

SAM#10.Fow: 17 mer; 5'-GCGGGCCAACGAGCACC-3'

From Bio-Synthesis

SAM#11. Fow: 19 mer; 5'-TCGGAGCACACACCAGCAC-3'

From Bio-Synthesis

SAM#12.Bak: 18 mer; 5'-CGACGAAAGCACCCAGGC-3'

From Bio-Synthesis

SAM#13.Fow: 16 mer; 5'-ACGCCAGAAGAACCGC-3'

From Bio-Synthesis

LAC2: 20 mer; 5'-TGAGAGAAGGGCTGTGACGC-3'

From Chander Raman

SAM3-2: 20 mer; 5'-GCATCATCACGCCCTACGAG-3'

From Chander Raman

SAM4-2: 20 mer; 5'-ATCAGGCTCAGTGGTCTTTG-3'

From Chander Raman

GALP3: 20 mer; 5'-CCCTGATAACTATGGCGATG-3'

From Chander Raman

GALP3.rev: 20 mer; 5'-TACTGGTAGGCGTCCTCGTA-3'

From Chander Raman

SAM3.rev: 20 mer; 5'-GAAGATGTTGGATGGGAAGG-3'

From Chander Raman

SAM4.rev: 18 mer; 5'-CGAGGCTGGCCAAGATGC-3'

From Chander Raman

Gal-UP.Forward: 18 mer; 5'-TTCGGTTTGTATTACTTC-3'

From Loyola Core Facility

λGT10GENE.F: forward primer: 27 mer;

5'-CTTTTGAGCAAGTTCAGCCTGGTTAAG-3'

From Gene Napolitano

λGT10GENE.B: backward primer: 24 mer;

5'-GGCTTATGAGTATTTCTTCCAGGG-3'

From Gene Napolitano

UPF1-5'-*Eco*RI primer: 29 mer;

5'-CGAATTCGAAATGGTCGGTTCCGGTTCTC-3'

From Bio-Synthesis

UPF1-3'- *Bsa*BI primer: 30 mer;

5'-ATTGATCAGTATCCCAGTTCGCATTTTTCG-3'

From Bio-Synthesis

SAM#3.Fow: 20 mer; 5'-GCTGATCCTTGTAGGCGACC-3'

From Loyola Core Facility

SAM#4.Bak: 20 mer; 5'-GGTCGCCTACAAGGATCAGC-3'

From Loyola Core Facility

SAM#5.Bak: 18 mer; 5'-CCTGGGACAGCTCCGGCT-3'

From Loyola Core Facility

SAM#6.Fow; 18 mer; 5'-GGACAGCTCGACGCGCAG-3'

From Loyola Core Facility

Peptides

Peptides used in this study are of two forms. First, multiple antigenic peptide (MAPS) (Ponsette et al., 1988), or COOH-terminal branched peptides, were synthesized by Ingrid Haas (University of Heidelberg, Germany). Second, linear, non-branched peptides synthesized by Bassam Wakim (Loyola University Macromolecular Core Facility).

UPF1#1; 16 mer; (GenBank #M76659:Codons 950 to 965)

NH_3-ARELQREEQKHELSKD - Poly-K

UPF1#2; 16 mer; (GenBank #M76659:Codons 920 to 935)

NH_3-KSAFSQKQNRNEIDDR - Poly-K

UPF1#3; 14 mer; (GenBank #M76659:Codons 635 to 648)

NH_3-EVQYRMNPYLSEFP - Poly-K

NORF1-pep#1 (Lab name: SAM-PEP#1); 18 mer; (Codons 106 to 122)

NH_3-EEDEEDTYYTKDLPIHAC-COOH

NORF1-pep#2 (Lab name: SAM-PEP#2); 24 mer; (Codons 214 to 237)

NH_3-ASQSSLKDINWDSSQWQPLIQDRC-COOH

Plasmids and DNA Probes

Cloning Vectors and Plasmids

Plasmid clones are indicated by name and by file number in the Chicago books in the laboratory of Dr. Hans-Martin Jäck.

pSP73 (plasmid clone: Chicago # 499)	Promega, Madison, WI
pUPF1 (plasmid clone: Chicago # 443)	Dr. M. Culbertson (Leeds et al., 1993)
pUPF1-D1 (plasmid clone: Chicago # 490)	Dr. M. Culbertson (Leeds et al., 1993)
pUPF1-D4 (plasmid clone: Chicago # 491)	Dr. M. Culbertson (Leeds et al., 1993)
pHβ-Apr-1-neo (plasmid clone: Chicago # 412)	From Ron Corley (Gunning et al., 1987)
p73-UPF1Δ0.8 (plasmid clone: Chicago # 497)	This work
p73-UPF1/-5'3' (plasmid clone:	This work

Chicago # 561)	
pUPF1-5' end (plasmid clone:	This work
Chicago # 562)	
pUPF1/β-actin (plasmid clone:	This work
Chicago # 564)	
pUPF1-D1/β-actin (plasmid clone:	This work
Chicago # 609)	
pR-TRAPα (p5.1.1.X and p7.1.1.X)	This work
(plasmid clone: Chicago # 533 and 533)	
p2.0-*Hind*III (plasmid clone:	This work
Chicago # 505)	
p6.4-*Eco*RI (plasmid clone:	This work
Chicago # 525)	
pNORF1 (p11.2 gene, 3.6-kb)	This work
(plasmid clone: Chicago # 579)	
pNORF1 (p15.1.1.1. gene, 5.5-kb)	This work
(plasmid clone: Chicago # 610)	
pR13609 (plasmid clone:	Human EST sequencing project
Chicago # 592)	Boguski et al., 1993
pH11830 (plasmid clone:	Human EST sequencing project
Chicago # 593)	Boguski et al., 1993
pH11167 (plasmid clone:	Human EST sequencing project

Chicago # 594)	Boguski et al., 1993
pH15581 (plasmid clone:	Human EST sequencing project
Chicago # 595)	Boguski et al., 1993
pNORF1/7Z (pGem7Z+3.5kb NORF1)	This work
(plasmid clone: Chicago #599)	
pβ-NORF1 antisense	This work
(plasmid clone: Chicago # 611)	
pβ-NORF1 sense	This work
(plasmid clone: Chicago # 612)	

DNA Probes

rabbit GAPDH (plasmid clone:	1.2-kb *Hind*III fragment from pR-
Chicago # 265)	GAPDH (Applequist et al., 1995)
yeast UPF1	3.2-kb *Eco*RI/*Xba*I fragment from
	pUPF1 (a gift from M.Culbertson)
yeast UPF1 RNA helicase region	1.3-kb *Dra*I fragment from pUPF1
yeast UPF1 5'-end region	1.4-kb *Eco*RI/*Kpn*I fragment from
	pUPF1
yeast CAN1	1.3-kb *Hind*III fragment from
	pTLC-1 (Broach et al., 1987)
mouse μ cDNA (plasmid clone:	1.4-kb *Apa*I/*Sma*I
Chicago # 254)	

human cDNA fragment R13609	1.6-kb *Hind*III/*Eco*RI or *Hind*III/*Not*I fragment
human cDNA fragment H11830	1.4-kb *Hind*III/*Eco*RI or *Hind*III/*Not*I fragment
human cDNA fragment H11167	1.7-kb *Hind*III/*Not*I fragment
human cDNA fragment 11.2	3.5-kb *Xho*I fragment from p11.2.2 and p11.2.1
human cDNA fragment 5' end of NORF1	1.3-kb *Xho*I/*Sal*I fragment from pNORF1/7Z
human cDNA fragment 3' end of 11.2	600-bp *Bst*XI/*Xho*I fragment from NORF1 pNORF1/7Z

Analysis of RNA

Isolation of RNA from Mammalian Cells

Solutions and Reagents

guanidium isothiocyanate (GIT) homogenization buffer	Mix 94.53 g guanidine isothiocyanate (4 M final), 1.67 ml 3 M Na-acetate, (pH 6.0); and add dH_2O to 200 ml. Add 8.35 µl/ml β-ME before each use.

cesium chloride buffer	Mix 95.97 g CsCl (5.7 M); 0.83 ml 3 M Na-acetate, (pH 6.0); add dH_2O to 100 ml, and sterile filter.
DEPC-TE	Prepare TE using stock Tris-HCl, (pH 7.4); and 0.5 M EDTA using 0.1% DEPC-dH_2O.

Total RNA Isolation

Total RNA was isolated from cultured cells by either the GIT/cesium chloride method or by the Qiagen RNA preparation method (both described below).

Guanidinium Thiocyanate/Cesium Chloride Method

Total RNA was prepared from cultured cells with GIT followed by centrifugation through a cesium chloride (CsCl) solution (Chirgwin et al., 1979). Briefly, 1-2 X 10^7 cells were removed and washed with ice-cold sterile phosphate buffered saline solution (Dulbecco's PBS, Cat#21-031-LV, Gibco-BRL). The cells were transferred in 1 ml of PBS to a 15 ml-Falcon tube pelleted, decanted, and resuspended in residual liquid by tapping the tube vigorously. Cells were then resuspended in 2 ml of GIT homogenization buffer. The resulting cell homogenate was layered on 2 ml of CsCl solution in a clear 13 X 51 mm thin

wall/polymer open top ultracentrifuge tube (Nalgene UltraTubes, Nalge, Rochester, NY). The tubes were balanced with GIT homogenization buffer and centrifuged with 35,000 rpm for 18-20 hr at 20°C in a Beckman SW50.1 rotor and Beckman Preparative Ultracentrifuge (Model L8-70, Beckman, Palo Alto, CA). After centrifugation the supernatant was aspirated until the CsCl layer was reached. CsCl was removed by gentle pipetting. The bottom of the tube was cut off with a razor blade, and the clear RNA pellet (3-5 mm in diameter) was resuspended in 3 X 100 μl of ice-cold DEPC-TE and transferred to an autoclaved, chilled, 1.5 ml microcentrifuge tube. The RNA was precipitated overnight at -20°C after the addition of 1/10 volume of 3 M NaAc, (pH 5.2); and 2.5 volume of ice-cold EtOH. The RNA was collected by centrifugation at full speed at 4°C for 2 hrs, washing with 70% EtOH, air-drying, and resuspended in DEPC-TE at concentration of 1-5 μg/μl.

Qiagen Method

The method was performed exactly as described by the manufacturer. All cell culture samples were disrupted by passing the GIT/cell resuspension repeatedly through a 20G$1_{1/5}$ gauge needle until foamy. The mixture was then processed as described in the RNeasy kit handbook.

Cytoplasmic RNA Isolation

Cytoplasmic RNA was isolated by the Nonidet-P40/phenol method (modified from Favaloro et al., 1980). Briefly, 5 X10^6 cells were pelleted, washed once with ice-cold Dulbecco's PBS, transferred to a microcentrifuge tube and lysed for 30 minutes on ice in 200 µl of cold lysis buffer (from 5 Prime-3 Prime, Inc., Boulder, CO; 0.14 M NaCl, 1.5 mM $MgCl_2$, 0.5% Nonidet-p40, 10 mM Tris, (pH 8.6)) supplemented with 0.05 U/ml RNasin (Promega, Madison, WI). The nuclei and unlysed cells were removed by centrifugation at full speed in a microcentrifuge at 4°C for 30 seconds. 175 µl of the supernatant was transferred to a new microcentrifuge tube. RNA was then extracted twice with 300 µl of phenol/chisam (1+1) heated to 65°C and once with chisam at room temperature. RNA was precipitated with 1/10 volume NaAc and 2.5 volume ice-cold EtOH. The RNA was collected as described above and resuspended in 25 µl of DEPC-TE.

RNA Isolation from Yeast Cells

Total RNA was isolated from yeast by the method of Elion and Warner, (1984). Briefly, 10 ml overnight cultures of yeast cells with an OD_{600} of 1-1.5 were pelleted, washed with ice-cold dH_2O, pelleted again, and decanted. The pellet was resuspended in 200 µl of LET-1% SDS (10 mM Tris-HCl, (pH 7.4); 0.1 M EDTA, 0.1 M LiCl, 1.0% SDS) and frozen at -70°C overnight. The yeast were thawed on ice, transferred to a cold micro-centrifuge tube, mixed with 120 µl of

autoclaved glass beads, and vortexed for 30 sec. 25 µl of phenol/chloroform (1+1) are added and vortexed again 4 X 30 seconds with the sample on ice between vortexings. 480 µl of LET-0.2% SDS (same as above only to a final concentration of 0.2% SDS) is added to the sample and mixed. The lysate is removed and extracted twice with 400 µl of TE equilibrated phenol, (pH 7.0); warmed to 65°C. Place the samples on ice for 5 minutes between each extraction. The aqueous phase is removed from the final extraction, precipitated at -70oC the addition of 1/10 volume of 3 M LiCl and 2.5 volume of EtOH. The precipitated RNA is collected by centrifugation at full speed for 1 hr at 4°C and the RNA is dissolved in 50 µl of DEPC-TE. The quantitation is performed as in section 2.14.

Quantitation of RNA

The concentration of RNA was determined by measuring the optical density (OD) at 260nm using the following equation:

$$\mu g / \mu l \text{ RNA} = OD_{260} \text{ X } 40 \text{ X dilution factor.}$$

The purity of RNA was determined by comparing the ratio of the absorbance of RNA at 260nm to that at 280nm. Samples in the range of 1.8 to 2.0 are considered relatively pure from protein contamination.

Northern Blot Analysis

Solutions

10 X MOPS	Mix 41.9 g MOPS (sodium base), 3.72 g sodium EDTA, 4.1 g sodium acetate, add 0.1% DEPC-dH_2O to 1 L and pH to 7.0 with glacial acetic acid.
5 X RNA loading buffer	12.5% Ficoll (Sigma) 0.4% bromophenol blue 1 X MOPS DEPC-dH_2O to final percentages
Gel overlay buffer	Mix 50 ml of 10 X MOPS, 89.3 ml formaldehyde (37% from Sigma), and add DEPC-dH_2O to 500 ml.
Deionized formamide (dFA)	Formamide (Boehringer Mannheim) was deionized with 5g per 100 ml AG 501-X8(0) mixed bead resins (Bio-Rad) by

	stirring slowly at room temperature for 1 hour, filtered through a coarse Whatman filter disk and stored at -20°C in aliquots.
1 M sodium phosphate buffer (NaP, (pH 7.0))	200 ml 1 M NaH_2PO_4 600-700 ml 1 M Na_2HPO_4 to 1 L with dH_2O, (pH 7.0).
Prehybridization solution	Mix 2 ml dH_2O, 200 µl 10% SDS, 10 ml dFA, 5 ml 20 X SSC, 2 ml 50 X Denhart's solution, 1 ml 1 M sodium phosphate buffer, (pH 7.0); 0.25 mg/ml denatured sheared salmon sperm DNA (from 10 mg/ml stock solution).
Hybridization solution	Mix 150 µl 10% SDS, 7.5 ml dFA (50%), 3.75 ml 20 X SSC, 300 µl 50 X Denhart's solution, 300 µl 1 M sodium phosphate buffer, (pH

	7.0); 3 ml 50% dextran sulphate, 0.3 mg/ml denatured sheared salmon sperm DNA (from 10 mg/ml stock solution).
Agarose Gel Solution	Mix 2.4 g agarose with 144.3 ml DEPC-dH_2O. Microwave the solution until the agarose is completely melted. Add 35.7 ml formaldehyde (37%), 20 ml 10 X MOPS, mix and pour.

Procedure

This procedure is that as described by Jäck and Wabl (1988) and the hybridizations were performed as described by Meinkoth and Wahl (1984).

Electrophoresis

The appropriate amount of RNA (1-10 μg) was mixed with 10 μl of deionized formamide (dFA), 3.5 μl of 37% formaldehyde, 2 μl of 10 X MOPS buffer, 2 μl of EtBr (1mg/ml), and filled with DEPC-dH_2O to a final volume of 30 μl. The mixture was heated to 65°C for 5 minutes and chilled on ice. 3 μl of 5 X RNA loading buffer was added to each sample and loaded into sample wells of a

formaldehyde agarose electrophoresis gel poured in a horizontal gel electrophoresis chamber (Model H3, BRL) submerged in overlay buffer. The left and right chambers were filled with 1 X MOPS buffer and electrophorised under constant voltage of about 40 V for overnight (~16 hours) until the bromophenol blue has migrated to the bottom of the gel.

Transfer of RNA

The RNA gel was photographed using an ultraviolet light transiluminator and a Polaroid camera, rinsed with dH_2O and 10 X SSC. Capillary transfer to a nitrocellulose membrane was performed overnight (BA85, 0.45 μm, Schleicher and Schuell, Inc., Keene, NH) in 10 X SSC (Figure 6). Standards as well as 18S and 28/26S RNA was labeled using a pencil. Filters were dried under a heat lamp for 20 minutes, baked under high vacuum at 80°C for 2 hrs.

Hybridization

Filters were prehybridized and hybridized with either an Omniblot system (ABN) or Hybaid brand hybridization oven according to the manufactures instructions. The prehybridization was performed in the prehybridization mix for over 1 hour at 42°C. The prehybridization was replace with the hybridization mix containing approximately 3 X 10^7 cpm of denatured ^{32}P-labled probe and 500 μg of denatured sheared salmon sperm DNA. Hybridization was carried out for 12-48 hrs at 42°C. After hybridization, the filters were washed once with 2 X SSC at

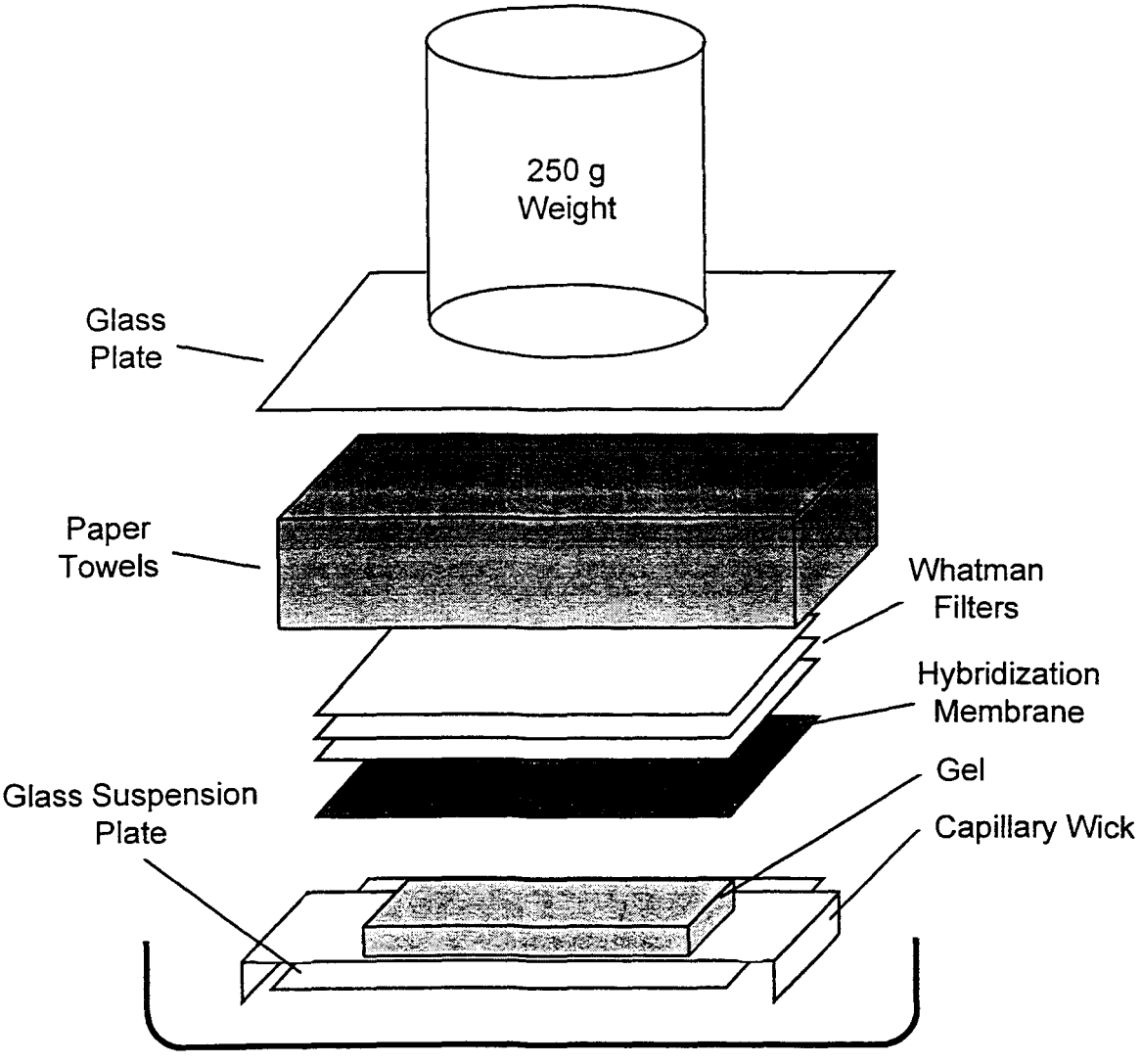


Figure. 6 Schematic diagram of a nucleic acid capillary transfer apparatus.

room temperature to remove free radioactive probe and then several times with 0.1 X SSC/0.1% SDS for 20-30 minutes at 52-54°C. The membrane was washed until the background detected by a Geiger counter was considered low. The washed blots were wrapped in plastic wrap and radioactivity on the blot was determined with a Betagen Radioanalytic Imaging System (Betascope 603 Blot Analyzer, Betagen Corp., Mountain View, CA). The blots were then wrapped in plastic wrap and exposed to X-ray film (Kodak X-OMAT AR film, Eastman Kodak Company, Rochester, NY) flanked by one intensifying screen (Fisher Biotech, Pittsburgh, PA) for varying times at -70°C before processing by a X-ray film processor (Model QX-60A, Konica, Tokyo, Japan).

The sizes of a RNA band was determined by measuring the distance between the loading well and the band and comparing it to that of known RNA molecular weight standards. The RNA size standard used was the RNA ladder and ribosomal RNAs.

To control for RNA loading on each lane, northern blots were re-hybridized with a 1.3-kb *Bam*HI/*Eco*RI fragment of rabbit GAPDH (Applequist et al., 1995).

RNase H Treatment of Total RNA

RNase H treatment of total RNA was used to identify regions where RNA/DNA hybrids were formed. The procedure was performed as adapted from and described by Qian et al.,1993.

Solutions

10 X RNase H buffer	0.2 M HEPES, (pH 8.0); 0.5 M KCl; 0.1 M $MgCl_2$
RNase H mix	Mix 2.5 µl of 10 X RNase H buffer, 2.5 µl of 10 mM DTT, 1.2 µl of RNase H (1 U/µl), and 8.8 µl of DEPC-dH_2O.

Procedure

Briefly, 10 µg of total RNA in 5 µl of dH_2O or TE was mixed with the following; 2 µl of oligo-(dT) primer (0.5 µg/µl; Promega, Madison, WI) or with 0.2 µg of a DNA oligonucleotide. This mixture was incubated for 30 minutes at 45°C. 15 µl of the RNase H mix was added, incubated for 20 minutes at 37°C. The mixture was extracted once with chisam, and the RNA was precipitated at -20°C with 1/10 volume 3 M NaAc, (pH 5.2); and 2.5 volume of ice-cold EtOH. The RNA was collected by centrifugation for 1 hr at maximum speed at 4°C, and subjected to northern blot analysis as described in section Northern Blot Analysis.

DNA Manipulations

General Solutions

20 X SSC	Mix 525.9 g NaCl, 264.7 g Citric Acid, and add 3 L dH_2O.
50 X Denhart's solution	Mix 1 g polyvinylpyrrolidone, 1 g of BSA, 1 g of Ficol (DL-400), and add 100 ml of dH_2O. Store Whatman filtered aliquots at -20°C.
0.5 M EDTA	Mix 186.1 g in 800 ml dH_2O, add NaOH (10N) until clear. Fill to 1000 ml with dH_2O.
2 M Tris-HCl, (pH 7.4)	Mix 12.114 g of Trisma-Base with 90 ml of dH_2O. pH to 7.4 with 12 M HCl. Fill to 100 ml with dH_2O.
3 M Sodium acetate	Mix 24.6 g of NaAc in 90 ml H_2O and pH to 5.2 by using glacial

	acetic acid. Fill to 100 ml with dH_2O.
TE buffer	Mix 5 ml of 1 M Tris-HCl, (pH 8.0); 1 ml of 0.5 M EDTA, and add 500 ml of dH_2O.

Salmon Sperm DNA: Resuspend 1 g of salmon sperm DNA in 90 ml of dH_2O and gently stir overnight. Sonicate the DNA solution on ice in 15 minute intervals. Test the average length of the DNA using TAE/agarose gel electrophoresis. Repeat the sonications until the average fragment length is between 1-3-kb and add SDS to 0.5% and proteinase K until 100 μg/ml. Incubate overnight at 42°C and then extract the DNA with TE equilibrated phenol, phenol:chisam (1+1), and then with chisam until the interphase is clear. Collect the aqueous phase and precipitate the DNA with 1/10 volume of 3 M NaAc and 2.5 volumes of EtOH. Collect the precipitate by centrifugation, wash with 70% EtOH, and resuspend in 90 ml of dH_2O. Quantitate the DNA and dilute to obtain a stock concentration of 10 μg/μl, aliquot and freeze at -20°C.

TE-saturated phenol/chisam: Melt phenol in 65°C waterbath. Add 8-Hydroxy Qunoline (Sigma Cat #H-6878) to a final concentration of 1 mg/ml phenol. Extract the phenol once with equal volumes of 1 M Tris-HCl, (pH 8.0); and 3 to 5 times until the pH of the TE phase reaches 7-8. Mix 1 part TE-equilibrated phenol with 1 part of chisam (chloroform/isoamyl alcohol at 25:1)

Amplification of Plasmid DNA

Plasmid DNA Preparations

Large preparations of plasmid DNA used for cloning, transfection and transformation were performed by the alkaline lysis as described by Sambrook and colleagues (1989) and also with a large scale plasmid kit from Qiagen as described by the Qiagen plasmid preparations manual (Qiagen Plasmid maxi kit, Cat#12162, Qiagen, Chatsworth, CA). Rapid, small scale plasmid preparations were performed as described by the Promega Magic minipreps kit (Cat#A7100, Promega, Madison, WI) according to the manufacturers instructions included with the kit.

Solutions and Media

LB-antibiotic media	2.5% LB 5 mM Glucose 100 μg/ml ampicillin, 15 μg/ml tetracycline, or 70 μg/ml kanamycin
Lysis buffer	1% glucose 10 mM EDTA, (pH 8.0) 25 mM Tris-HCl, (pH 8.0)

Lysozyme solution	30 mg/ml of lysozyme in lysis buffer
Alkali solution (prepare fresh)	1% SDS 0.2% N NaOH
Potassium acetate, (pH 4.8)	Mix 60 ml of 5 M KAc, 11.5 ml glacial acetic acid, and add 28.5 ml dH_2O.
Proteinase K buffer	50 mM Tris-HCl, (pH 8.0) 10 mM $CaCl_2$ (store -20°C)
Proteinase K stock	10 mg/ml of lyophilized proteinase K in proteinase K buffer (store -20°C)
RNase A buffer	10 mM Tris-HCl, (pH 7.5) 15 mM NaCl (store at -20°C)
RNase A stock	10 mg/ml of lyophilized RNase A in RNase A buffer (store -20°C)

Bacterial Strains

Escherichia coli (*E. coli*) strains HB101, JM109, or DH5α are from the lab stocks of the lab of Dr. Jäck. JM110 was obtained from New England BioLabs. All bacteria were made competent using a rubidium chloride/calcium chloride procedure (Sambrook et al., 1989). All bacteria containing plasmids were grown in either liquid culture or on agar plates in the presence of the appropriate antibiotic. In order to select for the presence of the DNA fragment disruption of the *E. coli* β-galactosidase α-peptide gene present on some plasmids, they were propagated in JM109 or DH5α were grown on Luria Broth (LB) agar plates (1.5% Bacto-agar in LB-media, (pH 7.0)) supplemented with 100 μg/ml ampicillin, 40 μg/ml X-Gal, and 5 mM IPTG.

Small Scale Preparation of Plasmid DNA (Miniprep)

A fresh, single colony was inoculated into 5 ml of LB-media+glucose containing the appropriate antibiotic. The cultures were shaken vigorously overnight at 37°C. 1.5-3 ml of the cultures were pelleted by centrifugation and the plasmids were isolated from the bacteria pellet with Magic mini prep kit (Promega, Madison, WI) according to the manufacturer's instructions.

Large Scale Preparation of Plasmid DNA

Alkaline Lysis Method

2.5 ml of an overnight culture was inoculated into 250 ml of LB-media +glucose medium containing appropriate antibiotic in a 1 L Erlenmeyer flask. The culture was shaken vigorously at 37°C until it reached an OD_{550} of 0.6-0.7. Bacteria were pelleted by centrifugation in a swinging bucket rotor in a Beckman table top centrifuge (Model GPR) at 3,200 rpm for 20 minutes at 4°C. The bacterial pellet was resuspended in 4 ml of lysis buffer. 1 ml of freshly prepared lysozyme was then added to the suspension. These cells were transferred to a 50 ml high speed centrifuge tube and incubated for 5 minutes at room temperature and 5 minutes on ice. The bacteria were lysed by the addition of 12 ml of alkali solution and subsequent incubation for 10 minutes on ice. Proteins were then precipitated by the addition of 9 ml of ice-cold potassium acetate solution for 20 minutes on ice. The solution was gently mixed, balanced, and then centrifuged in a Sorvall SS34 rotor at 12,000 rpm at 3°C for 30 minutes. The decanted solution was transferred into a new 50 ml tube and the DNA was precipitated with 1 volume of cold isopropanol for 20 minutes and collected by centrifugation in a Sorvall SS34 rotor at 15,000 rpm at 3°C for 30 minutes. The DNA pellet was partially dried by placing the tube in a Speed-Vac vacuum evaporator (Savant, Farmingdale, NY) and the pellet was resuspended in 2 ml TE. To eliminate the contaminating RNA the DNA was incubated with addition of

RNase A. 50 μl of RNase A was added and incubated at 37°C for 15-30 minutes. To remove contaminating proteins 100 μl of 10% SDS was added followed by incubation with 40 μl of proteinase K at 42°C for 1 hour. The treated DNA in TE was extracted 3 times with phenol/chisam and once with chisam. DNA was precipitated with 1/10 volume of 3 M sodium acetate, (pH 5.2); and 2.5 volume of ice-cold EtOH for 20 minutes to overnight at -20°C. DNA was pelleted a Sorval SS34 rotor with 12,000 rpm at 4°C. The DNA was washed with 70% ethanol, dried in a Speed-Vac vacuum evaporator, and dissolved in 250 μl of TE. 1 μl of this DNA was quantitated by restriction enzyme digestion as described in section Quantitation of DNA.

Qiagen Method

Large scale plasmid isolation by the Qiagen method was carried out using 100 ml bacteria cultures grown overnight in LB-media+glucose with antibiotics according to the manufacture's instructions (Cat#12162).

λ Phage DNA Preparation

λ Phage DNA preparation was carried out according to Sambrook et al., (1989). Briefly, eluted phage particles were titrated and infected into the appropriate strain at a pfu of 5 X 10^7 phage per 10^{10} *E. coli* cells. The cells were grown in 500 ml of LB-media+glucose containing 10 mM maltose and 10 mM $MgSO_4$ prewarmed to 37°C. Concomitant growth of bacteria and phages should

occur, which results in lysis and a clearing of the culture after 9 to 12 hours. To lyse residual bacteria, add 10 ml of chloroform to each flask and incubate for 10 minutes at 37°C with shaking. The culture is cooled to room temperature and DNase I and RNase A are added to a final concentration of 1 μg/ml. The mixture is incubated for 30 minutes at room temperature. NaCl is slowly added to the culture to a final concentration of 1 M (29.2 g per 500 ml of culture). The NaCl is dissolved by gentle swirling of the flask. The culture is incubated for 1 hour on ice and residual debris are removed using a GSA rotor with 11,000 rpm for 10 minutes at 4°C. Pool the supernatants in a clean flask and PEG 8000 is added to a final concentration of 10% w/v (i.e., 50 g per 500 ml of supernatant) and dissolve by slow stirring on a magnetic stirrer at room temperature. The phage particles are precipitated overnight on ice and pelleted by centrifugation in a GSA rotor with 11,000 rpm for 10 minutes at 4°C. Discard the supernatant thoroughly. Gently resuspend the pellet in 8 ml of phage buffer using a disposable plastic bulb pipette and extract the PEG from the suspension by adding an equal volume of chloroform and vortexing for 30 seconds followed by centrifugation in a Sorval SS34 rotor at 5000 rpm for 15 minutes at 4°C. 0.5 g of CsCl per ml of the phage solution is added and gently dissolved and layer onto CsCl step gradients prepared in a SW41 tube by adding the following CsCl solutions in phage buffer into the SW41 tube from the bottom up: 1.7 g/ml of phage buffer, 1.5 g/ml, 1.45 g/ml and then on top the 0.5 g/ml solution containing the resuspended bacteriophage pellets also in phage buffer. The gradient is

centrifuged in a SW41 rotor with 22,000 rpm for 2 hrs at 4°C. A bluish band of bacteriophage particles should be visible at the interface between the 1.45 g/ml layer and the 1.5 g/ml layer. The bluish band is collected by puncturing the tube, immobilized by a stand clamp, with a 21 gauge hypodermic needle followed by storage at 4°C. The λ DNA from intact particles are isolated by dialysis against a 1000 fold volume of 50 mM Tris-HCl, (pH 8.0); 10 mM NaCl, 10 mM $MgCl_2$. Prepare the dialysis tubing as follows. Cut 30 pieces of dialysis tubing each 20 cm long and boil them in 2% sodium hydrogen carbonate/1mM NaEDTA for 10 minutes. Add boiling stones and use a two liter glass beaker. Wash the tubing several times with dH_2O and boil for 10 minutes in dH_2O. Cool and wash the tubing at least 10 X with 250 ml of dH_2O and store in water with 0.1% azide at 3°C. Before use, rinse the tubing thoroughly with dH_2O and equilibrate for 5 minutes in dialysis buffer. The dialyzed bacteriophage is transferred into a centrifuge tube large enough so that the phage solution occupies 1/3 of the total volume. Add EDTA from a 0.5 M stock solution, (pH 8.0); to a final concentration of 20 mM, protease K to a final concentration of 50 μg/ml, incubate for 1 hour at 56°C, and cool the mixture to room temperature. Extract the phage solution once with equal volumes of TE equilibrated phenol, (pH 8.0); chisam, and chloroform. To separate the phases centrifuge the mixture at 3000 rpm in a table-top centrifuge at room temperature. Dialyze the phage DNA overnight at 4°C against three changes of a 1000 fold volume of TE, (pH 8.0). Remove the λ DNA from the

dialysis tubing and quantitate it as described in section Quantitation of DNA before use.

Enzyme Restriction of DNA

0.5-1.0 μg of plasmid and phage DNA were digested with 2-5 U of restriction enzyme in a 20 μl reaction volume in buffers provided by the manufacturer under reaction conditions recommended by the manufacturer. Digested DNA was analyzed on 0.8%-1.2% agarose gel as described in section DNA Agarose Gel Electrophoresis, and DNA fragments were isolated as described in section DNA Band Isolation from Agarose Gels.

DNA Agarose Gel Electrophoresis

DNA fragments were separated in 0.8%-1.2% TAE/agarose gels as described by Sambrook et al, (1989). The gels were prepared as follows. Dissolve 1 g of agarose in 125 ml TAE buffer (40 mM Tris-HCl, (pH 8.0); 20 mM sodium acetate, 2 mM EDTA) by boiling in a microwave oven, cooled to 60°C and add 4 μl of ethidium bromide (EtBr) stock solution (10 mg/ml of dH_2O). Mix gently and pour into a horizontal gel casting tray (Wide Mini-Sub DNA electrophoresis Cell, BioRad, Richmond, CA). Allowed the gel to solidify at room temperature for 60 minutes and submerge it in the electrophoresis chamber (Wide Mini-Sub DNA electrophoresis Cell, BioRad, Richmond, CA) containing 1 X TAE. Remove the comb before use. Add 1/5 volume of DNA loading buffer

(0.025% bromphenol blue, 2.5% Ficoll, 1 X TAE)to the DNA sample, heat for 5 minutes at 65°C, chill on ice and load 20-25 μl on the gel. Separate the DNA with 40-70 V until the bromphenol blue dye front reaches about 2/3 of the way through the gel.

Lambda (λ) DNA cleaved with restriction endonuclease *Hind*III, ϕχ DNA cleaved with *Hae*III and/or a 1 kb DNA ladder (all from Gibco BRL) were run simultaneously to serve as DNA molecular weight standards. DNA bands were viewed under an ultraviolet light box and photographed on Polaroid 667 film with a Polaroid Camera (Model DS34, Polaroid Corp., Cambridge, MA).

DNA Band Isolation from Agarose Gels

Digested DNA was size fractionated by agarose gel electrophoresis. EtBr DNA fragments were visualized using ultraviolet light. The appropriate fragments were isolated with a razor blade or scalpel and extracted from the gel by one of the methods described below.

Elu-Trap Method

DNA was isolated from the agarose piece by electroelution using a an Elu-Trap apparatus (Schleicher and Schuell, Keen NH, Cat#46178) according to the manufacturer's instructions. Briefly, the agarose piece was placed in the elution cell and electroeluted from the agarose with 120 V for approximately 2-4 hrs into a small elution chamber. The eluted DNA was removed from the

isolation chamber and precipitated with 1/10 volume of 3M NaAc and 2.5 volume EtOH overnight at -20°C. DNA was recovered by centrifugation and resuspend in TE to obtain approximately 1-2 μg DNA/μl.

Sodium Iodide (NaI) Method (Super-Band isolation)

The excised gel piece containing the DNA cut into small pieces and weighed. 2.5-3 volumes of 6 M NaI in dH_2O was added to the tube with the minced agarose and DNA. The mixture was incubated at 45°C for 10 to 20 minutes. The tubes are inverted to facilitate the dissolving of the agarose. 1 ml of Magic mini-prep resin (Promega, Madison, WI) was added to the melted agarose and incubated at room temperature for 15 minutes. The DNA fragments bound to the DNA binding resin were then processed according to the protocol provided by the manufacturer (Lit#TB117).

Quantitation of DNA

The concentrations of isolated DNA fragments, or plasmids, were estimated by comparing their EtBr-staining to that of DNA fragments of known amounts. Alternatively, optical densities at 260 and 280 nm were determined and the concentration wad calculated with the following equation:

$$\mu g / \mu l \text{ DNA} = OD_{260} \text{ X } 50 \text{ X dilution factor.}$$

Pure DNA is expected to yield a $OD_{260/280}$ of 1.8 to 2.0.

Dephosphorylation of DNA Fragments

To prevent self-ligation of vector fragments a nucleotide dephosphorylation reaction was carried out as follows. Pipette into a autoclaved microcentrifuge tube: digested DNA 20-30 µl, 10 X Calf Intestinal Phosphatase (CIP) buffer 5 µl, CIP enzyme 0.01 U/pmol DNA, and dH_2O to 50 µl total volume.

For 5'-overhangs, incubate the mixture at 37°C for 30 minutes, add another aliquot of diluted CIP, and incubate for another 30 minutes. For 3'-overhangs or blunt ends, the reaction mixture is incubated at 37°C for 15 minutes and then at 55°C for 15 minutes. Add another aliquot of CIP and incubate again at the same temperatures.

Inactivate the CIP enzyme by addition of 20 µl of 500 mM EGTA, (pH 8.0); followed by heat inactivation at 65°C for 45 minutes. CIP treated DNA is prepared for use by one phenol, (pH 8.0); extraction, one chisam extraction and EtOH precipitation. Precipitated DNA is resuspended in 10 µl of TE and quantitated for use.

Ligation of DNA Fragments

Solutions

10 X Ligation buffer	300 mM Tris-HCl, (pH 7.8); 100 mM $MgCl_2$, 100

	mM DTT, 5 mM ATP
10 X Klenow buffer	100 mM Tris-HCl, (pH 7.5); 500 mM NaCl 50 mM DTT
10 X T3 polymerase buffer	40 mM Tris-HCl, 6 mM $MgCl_2$, 10 mM DTT, 10 mM spermidine, 4 mM ATP, CTP, GTP, and UTP (pH 7.2 at 37°C)
10 X dNTPs	0.125 mM of each dNTP

Sticky-End Ligations of DNA Fragments

A 5-10:1 molar ratio of insert:vector DNA was used to obtain optimal ligation efficiency. Usually 0.1 μg of vector DNA was used in a 15 μl ligation reaction. One Weiss U of T4 DNA ligase (e.g. 1 Weiss unit is the amount of activity an enzyme can catalyze the ligation of greater than 95% of Lambda *Hind*III fragments of 1 μg of DNA at 16°C in 20 minutes) (Pharmacia or Promega) was added to each ligation mixture and ligation reactions were carried out at 15°C overnight (10-14 hrs).

Two-Step Ligations of DNA Fragments

Two-Step ligation is a procedure to ligate two fragments that are compatible only at one of their free ends.

Reaction 1. Ligation of compatible ends:

CIP-treated vector	0.5 μg
DNA fragment	5-10:1 excess over vector
10 X ligase buffer	2 μl
T4 DNA ligase	1 U (Weiss unit)
dH_2O	to final volume of 20 μl

Incubate overnight at 15°C and heat inactivate the ligase at 65°C for 10 minutes.

Reaction 2. Creation of compatible blunt ends.

For 5' overhangs:

DNA ligation mixture	20 μl
10 X Klenow buffer	3 μl
0.125 mM dNTPs	3 μl
100 mM DTT	1.5 μl
Klenow enzyme	1 U diluted in 1 X Klenow buffer
dH_2O	to final volume of 30 μl

Incubate at room temperature for 30 minutes.

For 3' overhangs:

3' the 10 X Klenow buffer is replaced with T4 DNA polymerase buffer and the Klenow enzyme is replaced with T4 DNA polymerase. Proceed as described for the Klenow reaction.

Reaction 3. Ligation of the blunt ends:

1. Ligation mixture

10 X ligase buffer	10 μl
50% PEG	10 μl
100 mM DTT	1 μl
T4 DNA ligase	1 U (Weiss unit)
dH_2O	to final volume of 100 μl

2. Add 40 μl of the ligation mixture to the Klenow/T4 DNA polymerase reaction 2 mix and incubate at 15°C overnight.

Transform the ligation mixture into competent *E. coli* cells as described in Transformations of *E. coli* With Plasmid DNA using 50 ng of ligated DNA.

Polymerase Chain Reaction (PCR) Amplification

PCR was performed with purified plasmid DNA or with complete λ phage particles. Either 1 ng of purified plasmid DNA or serial dilutions of λ phage

particles (eluted 2-6 hours) were used, as a template. PCR was performed with the reagents and protocols as described below

Solutions

10 X PCR buffer	Mix 1ml of 1 M Tris-HCl (pH 8.3), 5 ml of 1 M KCl, 250 µl of 1 M $MgCl_2$, 50 ml of 20% gelatin (BioRad), add dH_2O to 10 ml final volume. Store at -20°C in small aliquots.
10 X dNTPs (20 mM)	Mix 10 µl of 100 mM dATP, 10 µl of 100 mM dCTP, 10 µl of 100 mM dGTP, 10 µl of 100 mM dTTP, and add 500 µl of dH_2O. Store at -20°C in 50 µl aliquots.

Procedure

Pipette the following into a 0.5 ml autoclaved microcentrifuge tube:

Template DNA (about 1-5 µl)	X µl
1 ng of purified plasmid DNA or	
10 fold serial dilutions of eluted	

phage particles (eluted 2-6 hours)	
10 X PCR buffer	3.5 µl
10 X dNTPs	3.5 µl
forward primer (50 pmol/ml)	1 µl
backward primer (50 pmol/ml)	1 µl
Taq polymerase (5U/µl)	0.2 µl (1 U)
dH_2O	to 35 µl final volume

Overlay the mixture with 25 µl of mineral oil (Sigma, Cat# M-3516). Place the tubes in a thermal cycler (Perkin Elmer, Foster City , CA) and subject the tubes to the following reaction; 1 minutes at 95°C, 1 minutes at 68°C, 3 minutes at 72°C. The cycles were repeated 35 times, followed by a half hour at 4°C. Amplification products were analyzed and or purified on an agarose gel as described in DNA Agarose Gel Electrophoresis. Under certain conditions the annealing temperature of 68°C was lowered in order to allow primer annealing to the target DNA.

Cloning of PCR Products

Purified PCR products were cloned into the PCR cloning plasmid pGEM-T (Promega, Madison, WI) by the ligation methods described above and using the amounts of template as described by the manufacturer (Lit.#TB150).

Radio-labeling of DNA Probes

Nick Translation

Radioactive DNA probes were prepared by nick translation with a kit from Gibco-BRL according to the manufacturers instructions except that half of the volumes and α-^{32}P dCTP label were used. The labeled DNA was separated from unincorporated α-^{32}P dCTP nucleotides by gel chromatography on a G-50 Sephadex (Fine) spin column according to the protocol supplied by the manufacturer (Boehringer Mannheim) (Cat#1273-973). The radioactivity in 1 ml of column eluate was measured in a liquid scintillation counter (window 0-1500, Packard, Downers Grove, IL). The specific activity was calculated using the following formula:

$$\text{incorporation in cpm / } \mu\text{g DNA} = \frac{\text{cpm/}\mu\text{l x total eluate volume}}{\mu\text{g input DNA.}}$$

The average specific activities of nick translated probes using this protocol were 0.5-1 X 10^8 cpm/μg DNA.

5' End Labeling of oligonucleotides

Oligonucleotides were phosphorylated with T4 polynucleotide kinase in the presence of ^{32}P γ-labeled dATP. The reaction was carried out as described below.

100 pmol or 50 ng of	2 μl

oligonucleotide with free 5' ends	
10 X Kinase buffer "C" from	2 μl
Boehringer Mannheim	
(500 mM Tris-HCl,,100 mM	
$MgCl_2$, 1 mM EDTA, 50 mM	
DTT,1 mM spermidine, (pH 8.2	
at 25°C)	
^{32}P α-dATP (ICN >7000	4 μl
Ci/mmol SA)	
dH_2O	11 μl
T4 poly-nucleotide Kinase	1 μl
(10U/μl, Boehringer Mannheim)	

Incubate the mixture for 45 minutes at 37°C. After the reaction , fill to 50 μl with dH_2O and heat inactivate at 90°C for 5 minutes. Apply the reaction mixture to a G-25 Sephadex (Fine) spin column according to the protocol supplied by the manufacturer (Boehringer Mannheim) (Cat#1522990). The specific activity of the probe was calculated as described above. The average specific activities of labeled probes were between 1-2.4 X 10^9 cpm/μg oligonucleotide DNA.

DNA Sequencing

DNA sequencing was performed on double stranded plasmid templates with the Sequenase 2.0 sequencing kit from USB (Sanger et al., 1977). Plasmid templates were denatured by addition of 200 mM EDTA and 200 mM NaOH, incubation at 65°C for 2 minutes. 1/10 volume 3 M NaAc and 2.5 volume EtOH is added and the DNA is precipitated overnight at -20°C. Denatured plasmid DNA is resuspended in 5 μl of dH_2O, 0.5 pmol sequencing primer, and 2 μl of 5 X Sequenase Reaction Buffer is added and the volume is filled to 10 μl with dH_2O. The mixture is warmed to 65°C for 2 minutes, allowed to cool slowly to room temperature over a period of 30 minutes, and then chilled on ice. The Sequenase T7 DNA polymerase is diluted 1:8 in enzyme dilution buffer. To the annealed DNA and primer add 1 μl DTT, 2 μl labeling nucleotide mixture, 2 μl diluted Sequenase enzyme and 0.5 μl of ^{35}S α-dATP. The mixture is incubated at room temperature for 4 minutes. 3.5 μl of the sequencing reaction is added to 4 vials each containing 2.5 μl of either di-dioxy (dd) ddGTP, ddATP, ddTTP, or ddCTP respectively. The reaction is incubated for 5 minutes at 37°C and stopped by the addition of 4 μl of stop buffer. These sequencing reactions can be stored at -20°C for up to 4 days. Before loading onto the pre-heated sequencing gel as described in chapter DNA Acrylamide Gel Electrophoresis (Sequencing Gels), heat the samples to 90°C for 2 min.

DNA Acrylamide Gel Electrophoresis (Sequencing Gels)

Acrylamide gels for the separation of DNA subjected to Sanger dideoxy sequencing (Sanger et al., 1977) were prepared as described below.

Reagents

Acrylamide Stock Solution	Dissolve 40 g acrylamide and 2 g bis-acrylamide in 100 ml of dH_2O and sterile filter.
10 X TBE	Dissolve 284 g Trisma base, 165 g boric Acid, 120 ml 0.5 M EDTA, (pH 8.0); in 3 L of dH_2O
10% Ammonium persulfate (APS)	Dissolve 100 mg of ammonium persulfate in 1 ml dH_2O (can be used for up to 1 week)

Procedure

Clean 16 cm X 18 cm glass plates (Aladdin enterprise, San Francisco, CA) first with soap and hot water. Followed by dH_2O and finally with EtOH. Wipe with lint-free cloth and assemble with 0.6 mm spacers. Tape the sides and bottom of the glass plates and clamp together with large paper binding clamps

along each side. To prepare a 6% acrylamide denaturing urea gel, mix the following chemicals in a side-arm Erlenmeyer flask; 21 g Urea (Gibco-BRL), 7.25 ml acrylamide stock, 5 ml 10 X TBE, and 20 ml dH_2O. Mix the reagents, degas for 10 minutes, and add 400 µl of APS solution and 20 µl of TEMED (Sigma) with gentle swirling. Quickly pipette the mixture between the glass plates, insert the sharks tooth comb (Aladdin enterprise, San Francisco, CA) at the top of the gel, apply clamps near the top of the gel and allow polymerization to occur. Wrap the top of the polymerized gel in plastic wrap, apply 1 X TBE buffer to keep moist, and store overnight at room temperature. Carefully remove the comb and set up the glass plate in a electrophoresis apparatus (Aladdin enterprise, San Francisco, CA). Fill the top and bottom buffer tanks with 1 X TBE and insert the shark-tooth comb so that wells are formed. Prerun the gel with a constant voltage of 1000 V until the gel is warm and then increase the voltage to 1300-1800 V until the temperature of the gel is approximately 50°C. Flush the wells with fresh 1 X TBE, heat the samples to 85°C for 2 minutes, and immediately apply the sample to the gel. Run the samples until the xylene cyanole FF green dye band has just run off the bottom of the gel. Remove the thin glass plate, lay on top of the gel a 3 mm Whatman paper soaked in 10% acetic acid/ 10% methanol and incubate for 10 minutes. Remove any free acid/methanol solution by gentle blotting and then remove the Whatman (Note: the gel will stick to the filter paper). Cover the gel side of the filter paper with plastic wrap, dry the gel for at least 30 minutes at 80°C, and expose the gel to autoradiography.

Southern Blot Analysis of DNA

Solutions

Lysis Solution	Mix 680 µl 1 M $NaHCO_3$, 1.104 ml Na_2CO_3, 80 µl 0.5 M EDTA, 0.2 g N-lauroyl sarcosine (Na salt), add dH_2O to 40 ml and add 12 mg of proteinase K.
Solution A	0.25 M HCl
Solution B	0.5 M NaOH 1.5 M NaCl
Solution C	0.5 M Tris-HCl, (pH 7.4-7.5) 3.0 M NaCl
Southern Hybridization Mix	Mix 70 ml dH_2O, 20 ml 50% Dextran sulphate, 10 ml 10% SDS

DNA Isolation

Pellet 3 X 10^6 cells and wash once with ice-cold Dulbecco's PBS. Suspend the cell pellet and add 3 ml of lysis buffer, and using a 5 ml pipette, gently pipette the cell lysate up and down 2-3 times to lyse cells. Incubate the cell lysate at 37°C for 3-4 hr while periodically mixing the lysate. Transfer the lysate to a 50 ml conical tube extract with phenol (TE equilibrated, (pH 7.0)), chloroform, and isoamyl alcohol at a ratio of 25:24:1. Mix gently for 5-10 minutes and then separate the phases by centrifugation at 2370 rpm in a Beckman table-top centrifuge. Repeat the extraction step one time. Extract once with chloroform:isoamyl alcohol alone (24:1) and transfer the aqueous phase into a sterile 25 ml Erlenmeyer flask with 0.2 volume 11 M ammonium acetate. Add 2.5 volume 95% EtOH and mix the flask gently until DNA precipitates. Spool the DNA onto a Pasteur pipette and thoroughly wash the DNA in 70% EtOH. Dissolve the precipitated DNA at 37°C in 400 µl of dH_2O. Add 4 µl of RNase at 10 mg/ml and incubate at 37°C for 30 minutes. Extract the aqueous mixture as above. Precipitate DNA in a microcentrifuge tube by the addition of 1/10th volume of 3 M NaAc and 2.5 volumes of EtOH, pellet, wash, and resuspend the DNA in 400-600 µl of TE and incubate at 4°C overnight.

Restriction Digestion of DNA

Digest approximately 10 µg of DNA in appropriate restriction enzyme buffer as described in section Enzyme Restriction of DNA. Extract the digested

DNA twice with equal volumes of phenol/chloroform (1+1) and once with chloroform. Precipate and wash the DNA as described above and resuspend in 7.5 μl of 10 mM $NaPO_4$, (pH 6.8); in a volume suitable for application to TAE/agarose gel electrophoresis. Heat the sample at 37°C for >30 minutes, add 5 X DNA loading buffer, heat to 65°C for 5 minutes, chill on ice. Apply the sample to a 0.7% TAE/agarose gel with 30 V for 16-18 hr in 1 X TAE.

Transfer of DNA

After completion of the electrophoresis soak the gel for 15 minutes in solution A, 30 minutes in solution B, and in solution C for 45 minutes. Transfer the DNA to Gene Screen Plus (DuPont, Boston, MA) as described for RNA and then dry the membrane for 30 minutes at 37°C.

The filter is then prehybridized with the hybridization solution at 65°C and then hybridized at 65°C overnight in the same solution containing radiolabled probe boiled for 5 minutes with 100 μg of sheared salmon sperm DNA. The membrane is washed using high stringency conditions (68°C, 0.1 X SSC/0.1% SDS) for 10 minutes until washing is sufficient. The blot is then dried and put to X-ray film at -70°C backed with a fluorographic enhancing screen.

Bacterial Strains and Manipulations

Preparation of Transformation Competent Bacterial Cells

Solutions

Solution A	10 mM MOPS, (pH 6.5)
	10 mM RbCl
Solution B	10 mM MOPS, (pH 6.5)
	50 mM CaCl
	10 mM RbCl

Solutions A and B are filter-sterilized by passage through a 45 μm filter and stored at 4°C.

Procedure

The bacteria to be made competent are streaked from a frozen stock onto a LB-media plate. A single colony is inoculated into 5 ml of LB-media containing 5 mM Glucose (LB-media+glucose) and shaken vigorously overnight at 37°C. 100 ml of LB-media is innoculated with 2.5 ml of overnight culture. The culture is grown at 37°C until the OD_{600} is 0.13-0.15. The cells are centrifuged for 5 minutes with 5,000 X g at 4°C in a sterile tube. The supernatant is decanted and the cells are resuspended in 50 ml of solution A. The cells are pelleted as described above. The cells are resuspended in 5 ml of solution B and then up to

50 ml with solution B. The cells are incubated on ice for 30 minutes and then pelleted as described above. The supernatant is decanted and drained thoroughly. The cell pellet is gently resuspended in 4 ml of solution B. 1 ml of 50% glycerol is added to the resuspended cells and 50 μl aliquots are frozen in small tubes at -70°C.

Transformations of *E. coli* With Plasmid DNA

25 μl of competent *E. coli* cells in a microcentrifuge tube and 50-500 ng of plasmid DNA, or 1-5 μl of ligation mixture were mixed gently and incubated on ice for 30 minutes. The mix was heat shocked in a 42°C water bath for 50 seconds and placed on ice for 2 minutes. 1 ml of SOC medium (2% Bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the heat shocked cells, and the mixture was incubated at 37°C for 45-60 minutes. Aliquots of the transformation mix were plated on LB-media plates containing 100 μg/ml ampicillin, 15 μg/ml tetracycline or 70 μg/ml kanamycin. Transformants were screened as described in the following section.

Screening of *E. coli* Containing Recombinant Plasmid Vectors

Blue/White Color Screening

To screen transformed *E. coli* strains, JM109 and DH5α, for the presence of β–galactosidase activity, 50 μl of 2% X-Gal (Boehringer Mannheim, Indianapolis, IN, turns blue in the presence of β–galactosidase) and 100 μl of 100 mM isopropylthio-β-D-Galactoside (IPTG) (Boheringer Mannheim, Indianapolis, IN, induces promoter activity and expression of β–galactosidase α-peptide) were used. If the plasmid contains a DNA insert within the multiple cloning site, its presence will disrupt the α–peptide coding region and thus any α–peptide activity. To prepare media to detect β–galactoside activity within an *E. coli* strain containing a recombinant plasmid, the color inducing reagents were spread on 1.5% agar/LB-media plates containing an appropriate selective antibiotic (blue/white plates) for 30 minutes before transformed bacteria were plated. Recombinant plasmids are detected by their white color (lack of β–galactosidase activity due to recombinant DNA within the coding region of the α–peptide gene) while non-recombinant colonies (active β–galactosidase activity and active α–peptide) were blue.

Bacterial Colony Lift

To identify bacterial colonies that carry recombinant plasmids, the following protocol was adapted and modified from Buluwela et al., (1989). Briefly, LB-media plates containing bacterial colonies were cooled to 3°C before use. A nylon membrane (HyBond-N, 0.45 μm, X 82mm, Cat#RPH.82N,

Amersham, Arlington Heights, IL) was carefully laid onto the cold bacteria colonies and incubated for 2 minutes. To mark the orientation of the filter on the plate, filter and agar were punctured with a needle. The filter was removed and placed bacteria side up for 2 minutes on a Whatman filter soaked in denaturation solution (2 X SSC and 5% SDS). The filters were then placed on Whatman filter paper and microwaved on high power (100%) for 2-2.5 minutes. The filters were then soaked in 2 X SSC and the bacterial debris were gently rubbed off. The filters were prehybridized in a solution containing 0.5 M $NaPO_4$, (pH 7.0); 1% BSA (Boehringer Mannheim, Indianapolis, IN), 7% SDS, 1 mM EDTA at 65°C for at least 1 hour. 2 $X10^6$ counts of denatured ^{32}P-labeled DNA probe were added to the prehybridization mix and incubation was carried out overnight at 65°C. The filters were washed first with 2 X SSC at room temperature for 2-5 minutes and then with 0.1 X SSC/0.1% SDS at 55°C until the filter that serves as a negative control (bacteria transformed with vector alone) is clear of radiation. The punctures in the filter were labeled with radioactive ink and the filters were autoradiographed overnight using an enhancing screen at -70°C.

λ Phage Infections

Phage Strains and Host Strains

λ phage particles either from a purified plaque or as a whole library were infected into host bacterial strain as described here. The host strain used with each type of phage is indicated in Table 3.

Table 3. -- Bacteriophage and Host Strains Used

Phage Vector	Host Strain	Excision Strain	Reference
EMBL4	c600*hfl*	None	Woodcock et al., 1989
λgt10	LE392	None	Sambrook et al., 1989
λ ZapII	XL1-Blue MRF'	SOLR	Stratagene
λ Zap Express	XL1-Blue MRF'	XLOLR	Stratagene
λ FixII	XL1-Blue-MRA	None	Stratagene

Solutions

Solutions used in phage resuspension, infection, and plating are described below.

Phage buffer — Dilute 5 ml of 5 M NaCl, 1 ml of 1 M Tris-HCl, (pH 7.4); and 0.5 ml of 1 M $MgSO_4$ in 25 ml of dH_2O.

Top Agar	Autoclave 0.5 g of NaCl, 0.6 g of agarose, and 1 g of Bacto-tryptone in 100 ml of dH_2O. After autoclaving and cooling to 55°C add 5 ml of 1 M $MgSO_4$.

Phage Manipulation Protocols

Phage particles either undiluted or diluted in phage buffer were added to the appropriate bacterial strain that was grown to an OD_{550} of 0.6-0.7 in LB-media supplemented with 0.2% maltose and 50 mM $MgSO_4$. 100 µl of bacteria was used when the infection was plated on a 82 mm diameter plate and 300 µl if it was plated on a 137 mm diameter plate. Phage and bacteria were incubated together for 30 minutes at 37°C. Top agar warmed to 42°C (3 ml for a 82 mm plate and 7 ml for a 137 mm plate) was added to the bacteria/phage mixture inverted once and quickly spread on pre-warmed (37°C) plates. The plates were allowed to sit for 5 minutes at room temperature and then placed upside down at 37°C to allow plaques to form.

λ Phage Screening and Plaque Purification

LB-media plates containing lysogen plaques are screened for plaques containing the desired cDNA/DNA insert by using the following reagents and methods.

Solutions

Alkaline solution	Mix 60 g NaOH, 262.9 g NaCl, and add 3 L of dH_2O.
Neutralization solution	Mix 181.7 g Tris-HCl, 525.9 g NaCl, and add 3 L of dH_2O. pH to 7.4 using 12 M HCl.
3 X SSC/10% Dextran sulphate hyb solution	Mix 150 ml 20 X SSC, 100 ml 50 X Denhart's solution, 10 ml 10% SDS, 100 mg SS DNA (denatured), 100 g dextran sulphate and add dH_2O to 1 L
6 X SSC/10% Dextran sulphate hyb solution	Mix 300 ml 20 X SSC, 100 ml 50 X Denhart's solution, 10 ml 10 % SDS, 100 mg SS DNA (denatured), 100 g dextran sulphate, and add dH_2O to 1 L
2 X SSC/0.1% SDS	Mix 100 ml 20 X SSC, 10 ml 10 % SDS, and add dH_2O to 1 L

1 X SSC/0.1% SDS	Mix 50 ml 20 X SSC, 10 ml 10% SDS, and add dH_2O to 1 L
0.1 X SSC/0.1% SDS	Mix 5 ml 20 X SSC, 10 ml 10% SDS, and add dH_2O to 1 L

Procedure

Cool plates containing plaques to 4°C. Overlay plaques with a nitrocellulose filter of the appropriate size (82 mm diameter, BA85 [0.45 μm, Cat# 20440] or 132 mm diameter, BA 85 [0.45 μm, Cat# 20570] Schleicher and Schuell, Keene, NH) onto the plate until the filter is completely wet. Make alignment holes by puncturing the filter and agar plate with a needle. Lift filters from the plate with forceps and place plaque side up on a Whatman paper and dry for 5 minutes. Place the filters into alkaline solution for 1 minute and then transfer them into neutralization solution for 2 minutes. Remove the filters, dry them under a heat lamp, and then bake them at 80°C for 2 hrs. under high vacuum. Remove the filters and wet them in the appropriate hybridization solution without the dextran sulphate (either 3 X SSC mix for high stringency hybridization or 6 X SSC mix for low stringency hybridization). Remove the wetting solution and immerse the filters in the complete hybridization solution containing dextran sulphate for at least 1 hour at the appropriate hybridization temperature (64°C for high stringency [3 X SSC/10% dextran sulphate] or 55°C

for low stringency [6 X SSC/10% dextran sulphate]). Perform the hybridization overnight in the same solution and add 2 $X10^6$ counts of denatured ^{32}P-labeled DNA probe per ml. Wash the filters in the 2 X SSC/0.1% SDS solution at 55-58°C for low stringency screen and with 1-0.1 X SSC/0.1% SDS solution at 64-68°C for high stringency screen until the radiation on the negative control (filter with a lifted plaque or colony containing a known negative insert (e.g. GAPDH)) is minimal. Dry the filters, mark them with radioactive ink, cover them with plastic wrap, and expose them to X-ray film overnight against 1 enhancing screen at -70°C. Positive plaques were picked by excising a small agar piece by using the large or small end of a pasture pipette and placing the agar plug in 1 ml of phage buffer. The above procedure was repeated with eluted phage until all plaques screened positive with the radiolabled probe.

Plasmid excision from λYES phage by the *cre/lox* recombination system

λ-YES phage particle eluted from a plaque-purified single plaque and were used to infect the *E. coli* strain BNN132. This bacteria strain contains the *cre* recombinase protein that is able to recognize its cognate recombination site (*lox*) within the λ-YES phage DNA. The recombination process yields an ampicillin-resistant *E. coli* / yeast shuttle plasmid. The process of obtaining bacterial colonies containing the excised plasmid is described below.

The *E. coli* strain BNN132 was grown to an OD_{550} of 0.6 in selective media (LB, 0.2% glucose, 70 μg/ml kanamycin) to select for the presence of the

cre gene present within a kanamycin containing transposon. λ-YES phage particles are diluted in phage buffer and added to BNN132 at a ratio of 1 particle to every 3 bacteria (an OD_{550} of 0.2 contains 10^8 bacteria/ml). Incubate the phage and bacteria for 30 minutes at 30°C. Add 1 ml of selective media again, incubate the mixture for 1 hour at 37°C, and plate aliquots on LB-media+ampicillin agar plates to select for the presence of the excised plasmid. Incubate the plates overnight at 37°C and analyze the colonies as described.

Excision of Plasmid DNA from λ ZapII pahge by helper phage

Recombinant plasmids were excised from single cDNA phage clones isolated from Stratagene (La Jolla, CA) libraries λ-Zap Express or λ-Zap II. This was done exactly according to the Stratagene instruction manual (Cat#200253).

Hybridization with DNA oligonucleotides

Hybridization of nucleic acids supported on nitrocellulose membranes was performed as described by and adapted from M. Shulman, Univ. of Toronto.

Solutions

Hybridization mix	Mix 50 ml 20 X SSC, 4 ml 1 M Na_2PO_4, (pH 7.0); 14 g SDS, 40 ml 50 X Denhart's, and add dH_2O to 200 ml final volume

Wash solution	Mix 75 ml 20 X SSC, 100 ml 50 X Denhart's, 25 g SDS, 12.5 ml 1 M NaH_2PO_4, (pH 7.5); and add dH_2O to 500 ml final volume at 37°C

Procedure

Prehybridize Southern, northern or phage plaque blots on nitrocellulose at 47°C for at least 1 hour. Add ^{32}P-labeled probe (boiled 10 minutes then iced) directly to the prehybridization solution at a final concentration of 1 X 10^6 cpm/ml. Hybridize overnight at 47°C with gentle shaking. Wash the blot with the wash solution at 50°C for 10 minutes times until the negative control contains minimal background. Wash once with rinse solution (1 X SSC/1% SDS) at 50°C for 5 minutes and wrap the wet blot in plastic wrap and expose to film to test washing or dry the blot and put to film at -70°C with enhancing screen. Calculate the T_m of hybridization by the following equation: Add 2°C for each A or T and Add 4°C for each G or C. Hybridization should be at least 15-20°C below T_m.

Mammalian Cell Culture Techniques

Growth of Cells

All cell lines were grown in complete RPMI medium as described (Keyna et al., 1995). Breifly, all cell lines were maintained in RPMI 1640 medium supplemented with 10% (volume/volume) fetal calf serum (FCS, HyClone Laboratories), 4 mM L-Glutamine, 2.5 ml of penicillin-streptomycin solution (50 U/ml), 0.05 mM β-mercaptoethanol and 1 mM sodium pyruvate at 37°C or 30°C in a 5% CO_2-humidified incubator with copper lining (Model#B5060, Hereaus, South Plainfield, NJ). This medium is referred to as complete RPMI medium. Unless indicated otherwise, all cell culture reagents used were from Gibco-BRL.

Cell Lines Used For This Work

The cell lines used are described below.

Table 4. -- Cell Lines Used

Name	Type	Source	Reference
FH / LOCB 81.13.13	B cell hybridoma	Murine	Jäck et al., 1989
VXH / GAMO 12.8.10	B cell hybridoma	Murine	Jäck et al., 1989
NYC	B cell lymphoma	Murine	Jäck et al., 1992b
MORK	B cell hybridoma	Murine	Jäck et al., 1992a
U-937	Monocytic	Human	ATCC # CRL 1593
Raji	B lymphoma	Human	ATCC # CCL86
Jurkat	T lymphoma	Human	ATCC # TIB152
HeLa	Endothelial	Human	ATCC # CCL 2

Table 4. cont. -- Cell Lines Used

HA-VSMC	Heart Muscle Cell	Human	ATCC # CRL-1999
Cla	Glioma	Human	Established from a grade 4 neuroblastoma by L.C. Erickson, Loyola University
MC/CAR	Plasmacytoma	Human	ATCC # CRL8083
96-1	B cell hybridoma	Rabbit	Dr. Katherine Knight, unpublished

Harvesting of Cells

Cells were pelleted by centrifugation with 1100 rpm at 4°C for 5 minutes in a refrigerated table-top centrifuge (Silencer S-103 NA, , Rupp and Bowman, Tustin, CA).

Freezing and Thawing of Cells

To freeze cells, approximately 2.5 X 10^7 cells were pelleted, resuspended in 1.5 ml of RPMI freezing medium (RPMI 1640 supplemented with 30% FCS, 15% DMSO, and 0.05 mM β-ME), and transferred into a chilled cryogenic tube (Vangard CRYOS, Sumitomo Bakelite Co., Ltd., Japan). The frozen cells were kept at -70°C overnight and then transferred to liquid nitrogen for long-term storage. To thaw cells the frozen tubes were removed from liquid nitrogen and warmed in a 37°C water bath until most of the cells were thawed. The cells were

transferred to ice cold complete RPMI 1640 media, pelleted by centrifugation, and cultured in 15 ml of complete medium.

Counting of Cells

A aliquot of cells was removed from the flask and counted in a Neubauer hematocytometer chamber (Bright-Line, 0.1 mm, American Optical Co., Buffalo, NY). The cell number was determined by the following equation: Cells/ml = total number of cells in 1 of the 9 large counting fields X 2 X 10^4.

Transfection of Cells With Plasmid DNA

All cell transfections were carried out by electroporation as described (Jäck et al., 1992). 5 X 10^6 cells were collected and washed twice in ice-cold FCS-free RPMI medium and resuspended in 500 μl of the same medium. 5-20 μg of DNA (in approximately 20 μl of TE) is added and mixed gently by pipetting. The cell suspension is subjected to one electric pulse of 330 μF, 285 volts at low conductivity on ice with a Cell Porator (BRL, Gaithersburg, MD). The electroporated cells were immediately transferred into 10 ml of complete RPMI medium/20% FCS and grown for 2 days. To isolate stable transfectants, the cells were diluted in complete RPMI containing 0.8 μg/ml G418 (Geneticin, Gibco-BRL) to a density of 5 X 10^4 cells/ml media. 100 μl of the diluted cells were plated per-well in 10, 96 well plates. Wells were screened under the

microscope for cell clones between days 7-10 and fed with RPMI containing 0.8 μg/ml G418 at day 10.

Protein Analysis

Generation of Antisera Against Synthetic Peptides

Peptides deduced from the aa sequence of a polypeptide were synthesized, coupled to a carrier protein, and rabbits were immunized with the peptide / carrier conjugate. Linear polypeptides were coupled to a carrier molecule and used as an immunogen as described below. Branched polypeptides (Figure 7) were used directly as an immunogen due to their natural ability to prime an immune response without a carrier molecule.

Coupling of Non-branched Peptides to Carrier Molecules

Because small linear peptides of 15-30 amino acid residues are poorly immunogenic, they were coupled to a large carrier protein (keyhole limpet hemocyanin [KLH] or bovine serum albumin [BSA]) using commercially available pre-activated KLH and BSA (Pierce Co., Rockford, IL). The chemical reaction that leads to a covalent association of peptide and carrier is outlined in Figure 8. The coupling between peptide and carrier was performed with a antigen-carrier coupling kit (Pierce Co., Rockford, IL) according to the manufacturer's instructions included with the kit.

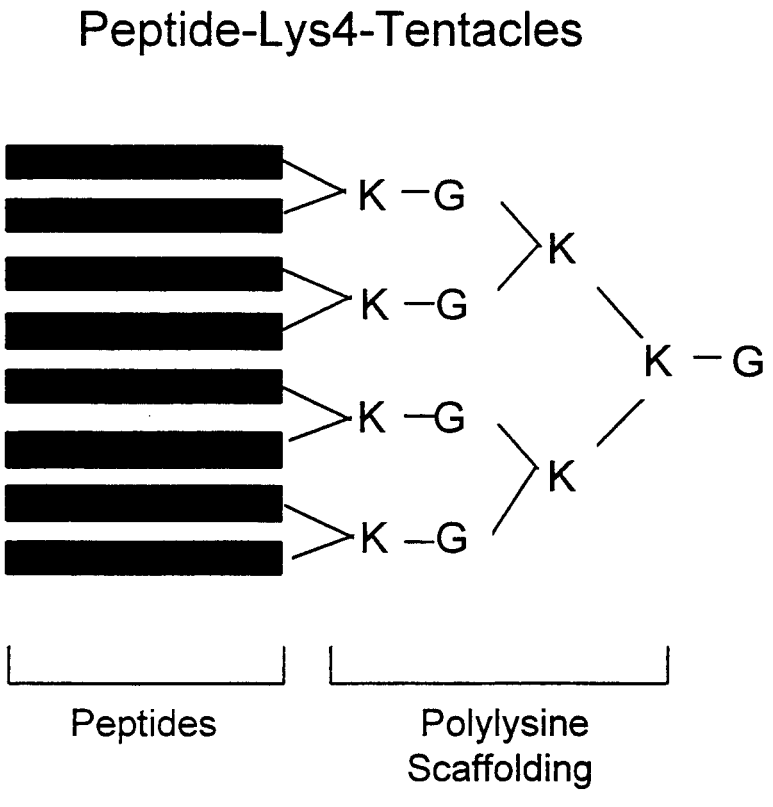


Figure 7. Schematic diagram of a multiple antigenic peptide.

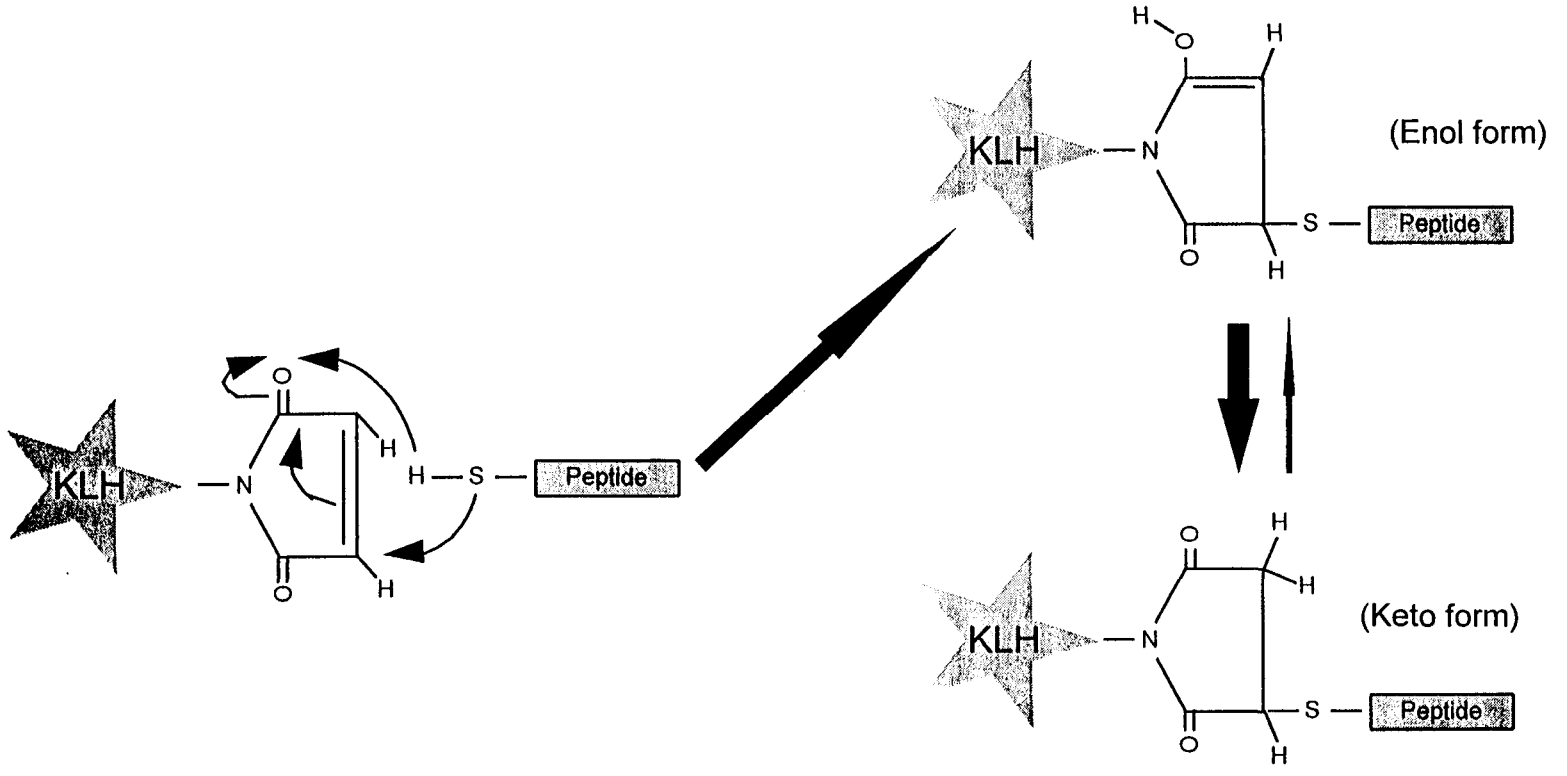


Figure 8. Schematic representation of the hapten / carrier linkage reaction using a maleimide activated carrier. The carrier KLH is represented by the star and a maleimide-reactive group by the chemical structure. The thiol group-containing peptide is represented by the grey box. Thick arrows represent the preferred direction of the chemical reaction. Curved arrows represent the direction and target of attacking reactive groups.

Immunization

Rabbits were either injected with carrier-coupled peptide or with branched peptides (antigen) as follows. The antigen is resuspended in PBS to a final concentration of 2 mg/ml and 1 ml of antigen is mixed with 500 μl of 0.9% NaCl and 1 ml of Complete Freund's Adjuvant (CFA). The emulsion is mixed using an emulsion mixer (5100 Mixer / Mill, Spex, Inc., Edison, NJ) for 5 minutes the mixture is put into a 3 ml syringe with a small spatula avoiding air bubbles whenever possible. Inject the whole mixture sub-dermal into the rabbits at different locations six times (by the rear legs, behind the neck and at their flanks) using a $20_{1/2}$ gauge needle. After 4-8 weeks boost the rabbits with the antigen in the following mixture: 100 mg antigen in PBS, 1 ml 0.9% NaCl, and 1 ml incomplete Freund's Adjuvant. If more boosting is required, repeat the procedure above.

One week after the second boost, the rabbits are bled from a small cut on the ear (no more than 30 ml from a healthy rabbit). The blood is collected in a 30 ml glass corex centrifuge tube and left at room temperature overnight (clotting occurs). Spin the mixture for 20 minutes at 2000 rpm in a table-top centrifuge and remove the cleared top layer of sera. Test the sera for antibody reactivity against the injected antigen.

Affinity Purification of Anti-peptide Antisera

To isolate peptide specific antibodies from the antiserum, isolated rabbit serum was applied to a peptide matrix column (Pierce Co., Rockford, IL). Briefly, a pre-activated amine-containing agarose matrix was mixed with the peptide containing at least one COOH group. The mixture was treated with a carbodiimide catalyst in order to facilitate the formation of an amine bond between the amine-containing matrix and the COOH containing peptide (Figure 9). The column is then washed of any free peptide. The prepared column is then incubated with rabbit sera allowing specific antibody-peptide interactions. Un-bound antibodys are washed from the column and then peptide-specific antibodies are released from the column by a 0.1 M Glycine (in dH_2O, (pH 2.5)).

ELISA Analysis of Antisera

Solutions

color reaction solution	Mix 10 mg Sigma 104 Phosphatase Substrate [Cat#104-0] in 10 ml of ELISA substrate buffer
ELISA substrate buffer	Mix 48.5 ml Diethanolamine, 400 mg $MgCl_2$+6H_2O, 100 mg NaN_3,

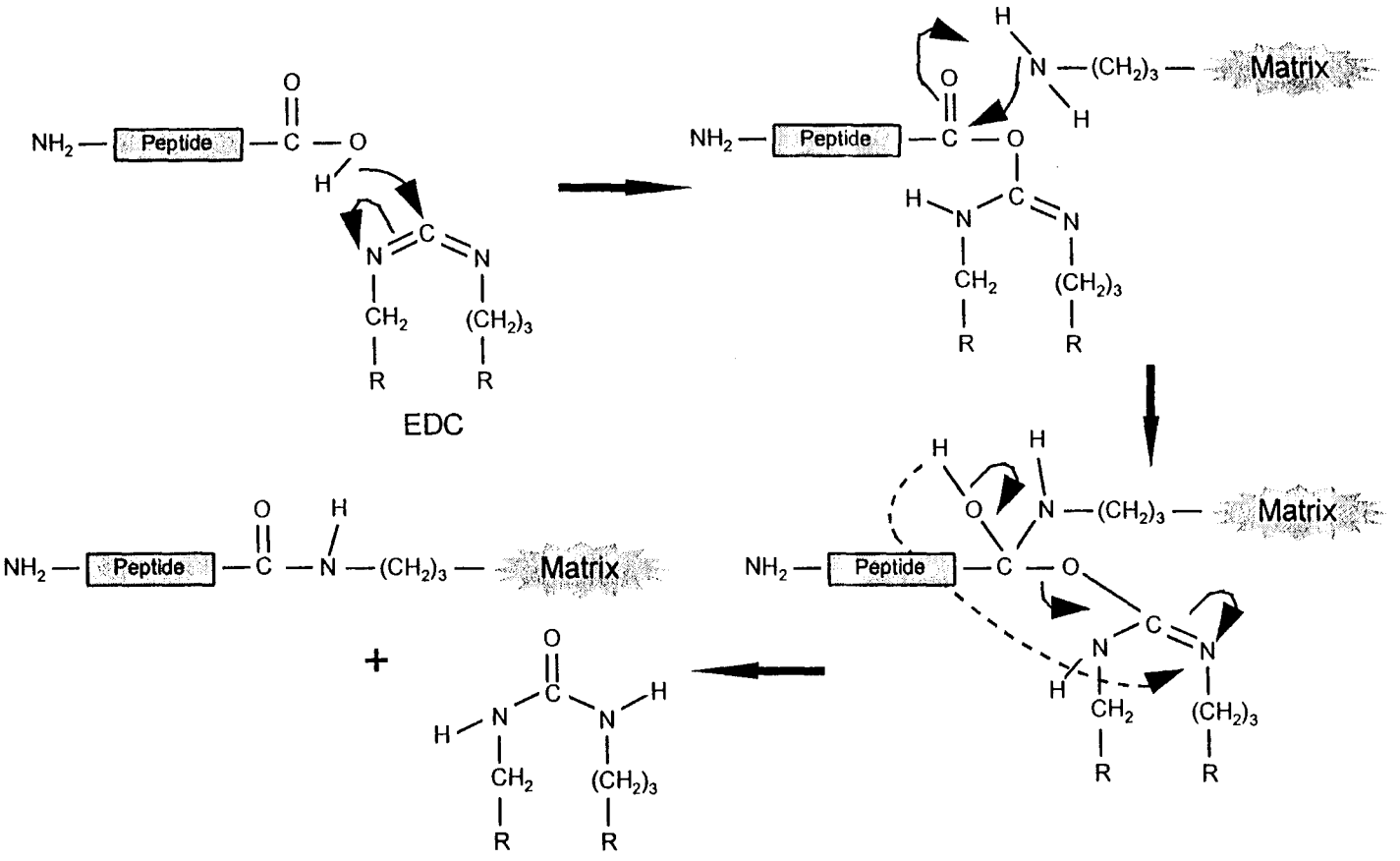


Figure 9. Schematic representation of the hapten / matrix linkage reaction using carbodiimide (EDC). The EDC facilitates the formation of an amide bond between a matrix amino group and a peptide carboxyl group (COOH). The matrix is represented by a star and the peptide with a free COOH group by the gray box. Thick arrows represent the preferred direction of the chemical reaction and the curved arrows represent the hypothetical movement of electrons and protons.

and add 500 ml dH_2O, pH to 9.8 with 12 M HCl.

Procedure

To determine the presence as well as the amount of specific antibodies, an enzyme linked immunoabsorbant assay ELISA assay was performed as described. 96 well plates (Falcon #3912-Test III) are first coated with target antigen at 10 μg/ml in PBS/0.1% NaN_3 overnight at 4°C. The wells were washed twice with antigen incubated plate is washed twice with PBST solution (1 X PBS, 0.05% Tween-20, 0.02% NaN_3) and then blocked with 200 μl of PBS + 1% BSA for 2 hr at 37°C or overnight at 4°C. After blocking, the wells were washed once with PBST, filled with serial dilutions of the antibodys solution and incubated for 2 hr at 37°C. The wells were washed 5 X with the PBST. Then 50 μl of the secondary alkaline phosphatase antibody (diluted 1:2000 in PBS + 0.1% NaN_3) is added and incubated for 2 hr at 37°C. The plates are washed 3 X with PBST 100 μl of color reaction solution is added. Incubate for 30 minutes and read in ELISA reader at λ_{450}.

Immunofluorescence Analysis of Cytoplasmic Proteins (CIF)

To detect cytoplasmic proteins, 1-5 X 10^4 cells in 200-300 μl of RPMI were centrifuged onto a glass slide at 1100 rpm for 2 minutes in a Shandon centrifuge (London, England). After briefly air-drying the pelleted cells, the slides

(and the cells on them) were placed in EtOH for 5 minutes at room temperature and rehydrated in PBSF solution (1 X PBS, 0.1% BSA, 0.1% NaN_3) for at least 20 minutes. For direct CIF the slides were then incubated with 10 µl of diluted fluorescent-labeled antibodies for 10 minutes at room temperature (Burrows et al., 1981). For indirect CIF, the slides were first incubated with 10 µl of primary antibody, washed 3 X with PBSF, and then incubated with 10 µl of diluted fluorescent-labeled secondary antibody. The slides were washed 3 X with PBSF and the cells were mounted under a glass coverslip using Cytoseal 60 (Stephens Scientific, Riverdale, NJ). Mounted cells were examined under a fluorescence microscope (Leica Photofluorescence Microscope, Model LABOVERT FS, Leitz Wetzlar, Germany).

Immunoprecipitation of Metabolically Labeled Polypeptides

Solutions and Reagents

RPMI labeling medium	Mix 500 ml methionine-free RPMI 1640 (Gibco-BRL), 50 ml of dialyzed Fetal calf serum (HyClone), 10 ml of 200 mM L-Glutamine (Gibco-BRL), 0.5 ml of 1 M sodium pyruvate, 5 ml of 500 U/ml Penicillin-Streptomycin

	(Gibco-BRL), and 2.5 ml of 10 mM β-ME.
10 X NET	Mix 8.76 g NaCl, 1.86 g of sodium EDTA, 6.06 g Trisma base, 1 g NaN_3, and add dH_2O to 100 ml. pH to 7.4 and store aliquots at -20°C.
0.1 M (PMSF)	0.1 M in ethanol, store at -20°C
1 X NET lysis buffer	Mix 10 ml of 10 X NET, 2.5 ml 20% Triton X-100 in water, and add dH_2O 100 ml. Add 1 µl of 0.1 M PMSF to every 100 µl of lysis buffer before use. Aliquot and store at -20°C.
Washed 10 % *Staphylococcus aureus*	*S. aureus* in PBS and NaN_3 was prepared as described in Bornemann (pH.D. thesis). Cells were collected, washed 2 X in *S.*

	aureus wash buffer and resuspended in the original volume of buffer.
S. aureus wash buffer	Mix 75 mg of methionine, 5 ml 0.5 M sodium EDTA, (pH 8.0); 12.5 ml 2 M Tris-HCl, (pH 8.0); 1 ml 10% NaN_3, 12.5 ml 20% Triton X-100, 25 ml 10% sodium DOC, 5 ml 10% SDS, 62.5 ml 4 M NaCl, and add dH_2O to 500 ml, pH to 8.3. Before each use add 1-1.2 mg/ml Ovalbumin (Chicken Egg, Sigma, St Louis, MO)
SDS sample buffer (1 X)	Mix 2.5 ml of 0.5 M Tris-HCl, (pH 6.8); 2 g glycerol, 1 ml β-ME, 200 µl 0.2% bromphenol blue, 5 ml 10% SDS, and add dH_2O to 20 ml.

Procedure

Metabolic Labeling of Cells

1-10 X 10^6 cells were starved for methionine for 1 hr in 1 ml RPMI labeling medium. 50 μCi/ml of Trans-[^{35}S] label (1076 Ci/mmol, ICN) was added and cells were incubated for 60-180 minutes in 5% CO_2 at 37°C. Cells were washed in ice-cold PBS and lysed with ice-cold NET lysis buffer (250 μl/ 1-5 X 10^6 cells) and incubated on ice for 20 minutes. Nuclei and un-lysed cells were removed by microcentrifugation at 4°C for 5 minutes. The lysate was then transferred to a new microcentrifuge tube and placed on ice.

Immunoprecipitation

Lysates were incubated for 4-16 hours at 4°C after the addition of the appropriate antisera or monoclonal antibody (approximately 5 μg antibody) to each 2 X 10^5 cells used (amount of the antibody may vary depending on the titer of the precipitating antibody used). In some cases where the primary antibody binds weakly or not at all to protein A, an appropriate secondary antibody was added which reacted with the first antibody and the incubation was carried out for 3 hrs at 4°C. Ab-Ag conjugates were precipitated by adding 100 ml of washed 10% *S. aureus* and incubated, the suspension was incubated for 30 minutes in ice. *S. aureus* was pelleted in a microcentrifuge tube, washed 2-3 times in *S. aureus* wash buffer and once with low salt washing buffer (50 mM

Tris-HCl, (pH 8.0)). *S. arueus* pellets were resuspended in 100 ml of 1 X SDS sample buffer, boiled for 3 minutes, and cooled in a water bath to room temperature. The *S. aureus* was pelleted and 50-75 ml of the blue solution was analyzed by SDS-polyacrylamide gel electrophoresis.

In Vitro Translation of Proteins

In vitro translation of proteins from RNA templates transcribed from cloned genes were performed with the TNT Coupled reticulocyte lysate system (Promega, Madison, WI) as recommended by the manufacturer. Briefly, 1-2 μg of linearized plasmid isolated from bacteria using magic mini-preps or from Qiagen was mixed with the following reagents;

1-2 μg of linearized plasmid DNA	X μl
TNT rabbit reticulocyte lysate	12.5 μl
TNT buffer	1 μl
T7 DNA polymerase	0.5 μl
^{35}S-methionine, cystine (Trans-label)	2 μl
10 mM amino acids (-methionine)	0.5 μl
20 U of RNasin (RNase inhibitor from Promega, Madison, WI)	0.5 μl
dH_2O to 25 μl final volume	

The *in vitro* translation mixture is incubated at 30°C for 120 minutes. 5 μl of the reaction was mixed with 1 X Laemmli reducing SDS sample buffer (see

section SDS Polyacrylamide Gel Electrophoresis (SDS/PAGE)), incubated for 15 minutes at 37°C. And analyzed by SDS-PAGE. Extra samples were frozen at -70°C for later use.

SDS Polyacrylamide Gel Electrophoresis (SDS/PAGE)

Proteins were separated by molecular weight on a discontinuous SDS polyacrylamide gel system described by Laemmli, (1970).

Reagents

acrylamide solution	Mix 40 g of acrylamide (40%), 1.07 g bis-acrylamide (1.07%), and add 100 ml of dH_2O.
8 X separating gel buffer (3 M Tris)	Mix 72.7 g of Trisma base, and add 200 ml of dH_2O. pH to 8.8 with HCl.
4 X stacking gel buffer (0.5 M Tris)	Mix 6.05 g of Trisma base, and add 100 ml of dH_2O. pH to 6.8.
10 % SDS	Mix 20 g of SDS with 200 ml of dH_2O.

10% Ammonium persulfate (APS)	Mix 100 mg of APS with 1 ml of dH_2O.
TEMED	Stock solution from Sigma
10 X Laemmli solution	Mix 121.2 g of Trisma base, 577 g glycine, 40 g SDS, and add 4 L of dH_2O.
separating gel buffer	Mix 3.75 ml of 3 M Tris-HCl, (pH 8.8); 0.3 ml 10% SDS, and add 30 ml of dH_2O.
1 X Laemmli sample buffer	Mix 2.5 ml of 0.5 M Tris-HCl, (pH 6.8); 2 g 100% glycerol, 1 ml β-ME, 200 µl 0.2% BPB, 5 ml 10% SDS, and add 25 ml of dH_2O.
3 X Laemmli sample buffer	Mix 7.5 ml of 0.5 M Tris-HCl, (pH 6.8); 6 g 100% glycerol, 3 ml β-ME, 600 ml 0.2% bromphenol

blue (BPB), 15 ml 10% SDS, and add 25 ml of dH_2O.

Electrophoresis

16 X 18 cm glass plates were washed, dried, and assembled with 1.5 mm plastic spacers and plate clamps in a Hoefer vertical gel casting stand (Model#SE6015). The gel solution (7.5 ml of acrylamide solution, 3.75 ml 3 M Tris-HCl, (pH 8.8); 18.135 ml dH_2O) was prepared in a 100 ml vacuum Erlenmeyer flask and degassed the solution under vacuum for 10 minutes. 300 µl of 10% SDS, 300 µl of 10% APS, and 15 µl of TEMED are quickly added and mixed by gentle swirling. The solution is then poured between the glass plates and then overlaid with a few ml of butanol saturated with Tris-HCl, (pH 6.8). The gel is allowed to polymerize overnight at 3°C. The stacking gel (2.5 ml 0.5 M Tris-HCl, (pH 6.8); 875 µl monomer solution, 6.525 ml dH_2O) is degassed for 10 minutes, and 100 µl of 10% SDS, 100 µl of 10% APS, and 6 µl of TEMED are added, mixed, and pipetted on top of the separation gel that has been washed with dH_2O. A 1.6 mm multi-well comb is carefully inserted between the plates and the gel is allowed to polymerize for 20-30 minutes. The comb is removed while applying 1 X Laemmli buffer to the top of the gel. The wells are flushed and filled with 1 X Laemmli running buffer. The samples in Laemmli sample buffer are boiled for 4 minutes, placed in room temperature water and loaded into the wells. The samples are separated with a constant current of 30 mA for 1

gel until the BPB band has reached the bottom of the gel. The glass plates are disassembled and the gel is either used for a western transfer (section Transfer of Electrophoreticaly Separated Proteins to Nitrocellulose Filters) or is enhanced and dried for fluorography. SDS-PAGE was also performed using a mini-gel system (Cat#67320). with the same solutions as described above.

Fluorography of Polyacrylamide Gels

To shorten film exposure time in the detection of radiolabeled compounds in polyacrylamide gels. Incubate the gel in ENTENSIFY-Part A solution for 30 minutes, then in ENTENSIFY-Part B (NEN Research Products, Boston, MA) for 30 minutes. Rinse the gel in dH_2O, place the gel on Whatman filter paper, dry the gel for two hours at 80°C and place it to film at -70°C using one enhancing screen.

Western Blot Analysis

Solutions

Transfer buffer	Mix 12 g of Trisma base, 57.6 g glycine, 800 ml methanol, and add 4 L of dH_2O.
10 X Ponceau S solution	2% Ponceau S dye

	30% trichloroacetic acid
	30% sulfosalicylic acid
Tris buffered saline	Mix 9.68 g of Trisma base, 116.96 g NaCl, and add 4 L of dH_2O. pH to 7.5 with 12 M HCl.
Blotto	Mix 5 g of dry, non-fat milk and add 100 ml of TBS.
antibody dilution solution	Mix 500 mg of dry, non-fat milk and add 100 ml TBS
Enhanced Chemiluminescence	ECL kit (Boehringer Mannheim)

Transfer of Electrophoretically Separated Proteins to Nitrocellulose Filters

Electrophoretically separated proteins were subjected to Western blot analysis (Beck-Engeser et al., 1987). Briefly, SDS gel was soaked for 10 minutes in 100 ml of transfer buffer. 3 Whatman pieces cut to the size of the gel were wetted in transfer buffer placed on a semi-dry transfer apparatus (Model #FB-SDB-2020, Fisher Biotech, Pittsburgh, PA) followed by the gel. The following sandwich was assembled. The gel is followed by a sheet of

nitrocellulose (BA85, 0.45 μm, Schleicher and Schuell, Keene, NH) cut to the size of the gel. Above the nitrocellulose is placed 3 additional Whatman sheets wetted in transfer buffer. Air-bubbles between the sheets were removed by rolling the sandwich with a pipette. Proteins were then transferred with a constant current of 10 mA-per cubic cm of sandwich for 2-2.5 hrs. The sandwich is then disassembled and the transfer of the proteins to the nitrocellulose is confirmed by Ponceau S staining for 10 minutes. The filter is then destained in TBS.

Immunodetection of Transferred Proteins on a Nitrocellulose Filter

The membrane is then destained and blocked in Blotto for greater than 1 hr, rinsed with TBS 2 X 15 minutes and incubated with the primary antibody (in antibody dilution solution) overnight at 4°C. After removal of the primary antibody the membrane is washed twice with TBS + 0.1% Tween 20 (BioRad, Cat# 170-6531) for 15 minutes and incubated with the secondary antibody (in antibody dilution solution) overnight at 4°C. Finally, the blots are washed twice with TBS for 15 minutes and subjected to enhanced chemiluminescence (ECL) as recommended by the manufacture (Boehringer Mannheim). Rainbow protein molecular weight marker were included to determine molecular masses of proteins and to control for protein transfer.

Yeast Strains, Growth and Manipulation

Yeast Strains

The *Saccharomyces cerevisiae* yeast strains used in this work are listed and discussed in Chapter 3.

Media and Reagents

YEPD (non-selective complete medium)

yeast extract (Difco)	10 g
bacto-peptone (Difco)	20 g
dextrose	20 g
dH_2O	to 1 L final volume

Autoclave aliquots. For growth on plates, add 25 g Bacto-agar per liter of YEPD.

Selective media

a) Drop-out medium (single selective or multiple selective medium)

dextrose	20 g
yeast nitrogen base w/o amino acids	6.7 g
appropriate 10 X drop-out	100 ml

stock solution	
50 mM inositol	1 ml
dH_2O	to 1 L final volume

Autoclave aliquots. For growth on plates, add 20 g Bacto-agar per liter of YEPD.

10 X drop-out stocks: The 10 X drop-out solution will not contain the amino acid for selection. For example *ura3* yeast will be grown in media without uracil to select for the presence of a plasmid containing the *URA3* gene. Add the following volumes of each amino acid supplement except the one that is being "dropped-out".

Table 5. -- Amino Acid Supplements Used in Yeast Drop-out Media

Supplement	Stock concentration	500 ml total
adenine	5 mg/ml	20 ml
arginine	20 mg/ml	5 ml
histidine	20 mg/ml	5 ml
leucine	10 mg/ml	30 ml
lysine	115 mg/ml	10 ml
methionine	20 mg/ml	5 ml
threonine	60 mg/ml	25 ml
tryptophane	10 mg/ml	10 ml
uracil	2 mg/ml	50 ml

Add 340 ml of dH_2O to the indicated amounts of amino acids and in place of the "dropped-out" supplement add the same amount of dH_2O. Mix thoroughly, make 100 ml aliquots, autoclave, and label according to the "dropped-out" supplement (e.g. 10 X Ura-).

b) Media supplemented with canavanine

dextrose	20 g
yeast nitrogen base	6.7 g
arginine drop-out stock	100 ml
50 mM insitol	1 ml
Agar	20 g
dH_2O	to 1 L final volume

Autoclave the mixture and cool to 57°C. Add the appropriate amount of stock canavanine solution to the media or aliquot of media to make an arginine dropout plate with the desired amount of canavanine. The average amount of canavanine to select a *can1* strain of yeast is 60 μg/ml.

Drop-out induction medium (Used to induce the transcription of genes driven by the *gal1-10* promoter).

Drop-out induction media

glycerol (80% stock)	37 ml
10 X drop-out stock	100 ml
yeast nitrogen base w/o	6.7 g

amino acids	
50 mM inositol	1 ml
dH_2O	to 1 L final volume

Autoclave the mixture and cool to 57°C before the addition of 10 ml of 100% EtOH. For plates add 20 g of agar per L of plates. Store at 4°C.

Introduction of Recombinant Plasmids Into Yeast

Solutions and Reagents

All yeast transformations described below were performed in the presence of carrier DNA (section DNA Manipulations and General Solutions) except that the average fragment length was approximately 2.0-0.7-kb.

Transformation buffer	Mix 2.4 g LiAc, 4 g PEG, (pH 5.0; MW 3550); 1.543 g DTT, and 100 ml of dH_2O. Freeze aliquots at -20°C.

For transformation, 50 μg of the DNA was boiled for 10 minutes and then put to ice. After this carrier was sufficiently cooled, 1 μg of the plasmid to be transformed was added.

Yeast Transformation

Yeast were transformed by the method of Chen et al., (1992). Resuspend 5 X 10^7 pelleted yeast cells in "One-step" transformation buffer. Mix the resuspended yeast with 1 μg of plasmid DNA and 50 μg of single-stranded carrier DNA (boiled salmon sperm) in a total volume of 100 μl. Incubated the mix at 45°C for 45 minutes and plate 10 μl and 90 μl of the suspensions directly onto selective medium and incubate the plates at 30°C for 3-4 days. Usually 20 μl and 80 μl were the plated cell volumes. The same protocol was used to transform DNA fragments into yeast.

Yeast Mating

The method used was adapted from a protocol by Rob Barrington (Dawes and Hardie, 1979). Yeast strains to be mated were grown on YEPD plates for 3 days to obtain healthy yeast. These yeast were used in the protocol below.

Generation of Diploid Yeast and Isolation of Yeast Spores

Media

Sporulation Plates

KAc (1% final)	10 g
yeast extract (0.1% final)	1 g
dextrose (0.05% final)	0.5 g

Bacto-agar (1.5% final)	15 g
dH_2O	to 1 L final volume

Procedure

Healthy yeast were picked from a YEPD plate using a toothpick. A 1 X 1 X 1 mm patch of each yeast (one strain a and one strain α) was mixed together with 5 µl of sterile dH_2O (patch mating). The mixed yeast were allowed to mate overnight at 30°C. The mixed yeast were streaked onto sporulation plates and incubated for 4-5 days at 25°C. Haploid spores were identified by suspension of a small amount of cells in a drop of water on a microscope slide and observed by light-field microscopy. The haploid tetrads will appear as clusters of four small spheres held within a barley discernible sphere. Spores were isolated by extraction in ether (Dawes and Hardie, 1979). Briefly, 5 small patches of sporulated yeast were picked and put into a microcentrifuge tube containing 100 µl dH_2O. The yeast were resuspended by brief vortexing. 300 µl of Ether was added to the mixture and vortexed once for 30 sec. The mixture was centrifuged for 5 minutes and decanted. This extraction procedure was repeated twice. The small pellet of material obtained after the final extraction contains separated spores (almost invisible). These spores were then resuspended in 100 ml of dH_2O and dilutions were plated onto the appropriate selective media to obtain haploid yeast of interest.

Replica Plating of Yeast

Yeast to be replica plated are grown on appropriate media. This master plate containing the strain or strains of interest is printed onto a velveteen square placed over a circular Plexiglas block by applying gentle pressure across the whole plate. A copy of this impression is transferred to plates made with all the relevant selective media including a plate identical to the one used as the master plate (positive control).

Storage of Yeast Strains

Yeast were stored as frozen stocks as described by Ausbel et al., (1990). Yeast were grown to late-log or early-stationary phase in appropriate media. 700 μl of the resuspenede culture was mixed with 300 μl of 50% glycerol and frozen at -70°C. Cells are revived by taking a fraction of cells from the frozen stock and plating on the appropriate media (usually YEPD).

CHAPTER IV

RESULTS

Rationale, Hypothesis and Overall Experimental Approaches

mRNAs containing nonsense codons (nonsense mRNA) encode truncated polypeptides that can interfere with the function of their corresponding full-length polypeptides. However, levels of most nonsense mRNAs are reduced in prokaryotes and eukaryotes when compared to that of the corresponding wild-type mRNAs (reviewed in Brawerman and Belasco, 1993). Genes that encode proteins responsible for the selective reduction of nonsense mRNA have been identified only in lower eukaryotes (reviewed in Maquat, 1995). One example is *UPF1*, a group I RNA helicase that is required to rapidly degrade cytoplasmic nonsense mRNA (Leeds et al., 1991, 1992; He et al., 1993). Since the half-lives of cytoplasmic nonsense β-globin mRNA (Cheng and Maquat, 1993; Belgrader et al., 1994) and immunoglobulin μ mRNA (Li and Jäck, unpublished observations) are decreased when compared to the corresponding wild-type mRNA, I hypothesized that mammalian cells contain a *UPF1*-like system that reduces levels of nonsense mRNA. To clone, or identify, a mammalian

gene required to reduce levels of nonsense mRNA, I chose the following approaches, all of which make use of the yeast *UPF1* gene.

- *First approach*

  Hybridize mammalian genomic and cDNA libraries with a yeast *UPF1* DNA probe.

- *Second approach*

  Transfect a *upf1* yeast strain with a mammalian cDNA expression library and rescue yeast transformants with a *UPF1*-positive phenotype.

- *Third approach*

  Determine whether the expression of a dominant-negative form of yeast Upf1p in a mammalian cell correlates with increased levels of nonsense mRNA.

- *Fourth approach*

  Search translated mammalian DNA and protein databases with the yeast *UPF1* amino acid sequence and determine whether identified genes are structural and functional homologues of yeast *UPF1*.

Because only the fourth approach led to the isolation of a mammalian *UPF1* homologue, I will describe and discuss this approach in detail. However, in the next three sections I will briefly review the results of the first three approaches. I will only discuss at length the data from the first approach, because it resulted in the isolation of a rabbit *TRAP*α cDNA that encodes a

protein thought to be involved in the translocation of nascent polypeptide chains across the ER membrane.

Hybridize Mammalian Genomic and cDNA Libraries With a Yeast *UPF1* DNA Probe.

The strategy to clone a mammalian homologue of the *S. cerevesiae UPF1* gene by hybridization was carried out using a number of straightforward molecular biological techniques and approaches. First, the yeast *UPF1* gene was used as a DNA probe to screen mammalian genomic DNA and cDNA libraries. To detect genes that have partial homology to *UPF1*, the hybridization was carried out under low stringency. Second, to determine whether the isolated genes are structurally homologous to the *S. cerevisiae UPF1* gene the nucleic acid sequence of the clone was determined. This approach, however, did not result in the isolation of a DNA clone with homology to *UPF1*. Instead, I cloned a cDNA encoding rabbit *TRAP*α, a protein that may be involved in the translocation of cytoplasmic proteins into the lumen of the endoplasmic reticulum (ER).

Isolation of a Rabbit Genomic Clone that Cross Hybridizes With the Yeast *UPF1* DNA Probe

To isolate a mammalian *UPF1* homologue, I screened a human complementary DNA (cDNA) phage library (Elledge et al., 1991) and a genomic

rabbit DNA phage library. The human library was chosen because a yeast expression plasmid containing the human cDNA insert can be easily excised from the phage and tested for its ability to complement a *upf1* allele yeast strain. The rabbit genomic DNA phage library (in EMBL4) was chosen for the following reasons. First, each gene should be represented in the library at least once, and second, filters containing about 3 X 10^5 lifted plaques were made available to me by Dr. Knight. The *UPF1* probe used to screen the libraries was a 1.3 kb-*Dra*I fragment (Figure 10.A). This DNA fragment contains the entire *UPF1* RNA helicase region. Mutations in this region can lead to dominant-negative forms of *UPF1* that, when transformed into wild-type *UPF1* strains, abolish the function of the wild-type *UPF1*, that is, nonsense mRNA has the same turnover rate as the corresponding functional mRNA. Therefore, a mammalian *UPF1* homologue may have conserved this important functional region, and thus, may contain enough nucleotide homologies that the human or rabbit homologue could be detected by using the *UPF1* helicase probe. The flow-chart in Figure 11 shows the individual steps of the hybridization approach and summarizes the major results. When I screened filters that contained 2.5 X 10^5 individual phage from the human HeLa cell cDNA phage library or the rabbit genomic phage library, I identified 2 genomic rabbit phage clones (Figure 10.B and Figure 11, box 2) and no HeLa cDNA clones that hybridized with the *UPF1* RNA helicase probe. To find the region of nucleotide homology within the two rabbit genomic phage clones and the *UPF1* RNA helicase probe, I proceeded to isolate total

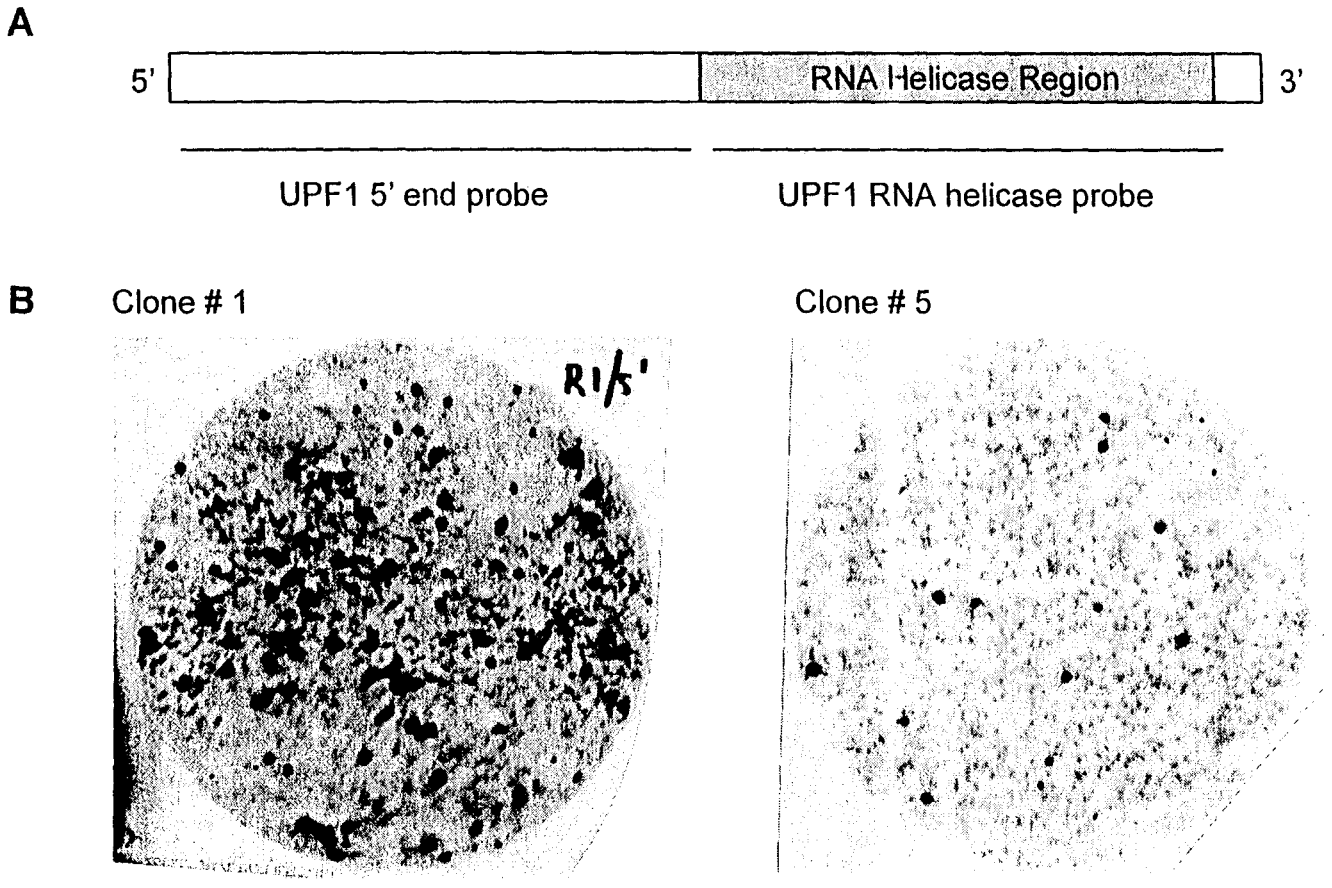


Figure 10. Schematic representation of the regions of the *UPF1* gene used as probes in library and Southern blot analysis. **(A)** The bar represents the *UPF1* ORF. The regions used as probes are shown below the UPF1 ORF. **(B)** Autoradiograph of rabbit genomic phage clones R1, and R5 after 1 round of plaque purification. Phage clones were hybridized with *UPF1* RNA helicase probe using low stringency hybridization conditions as described in materials and methods.

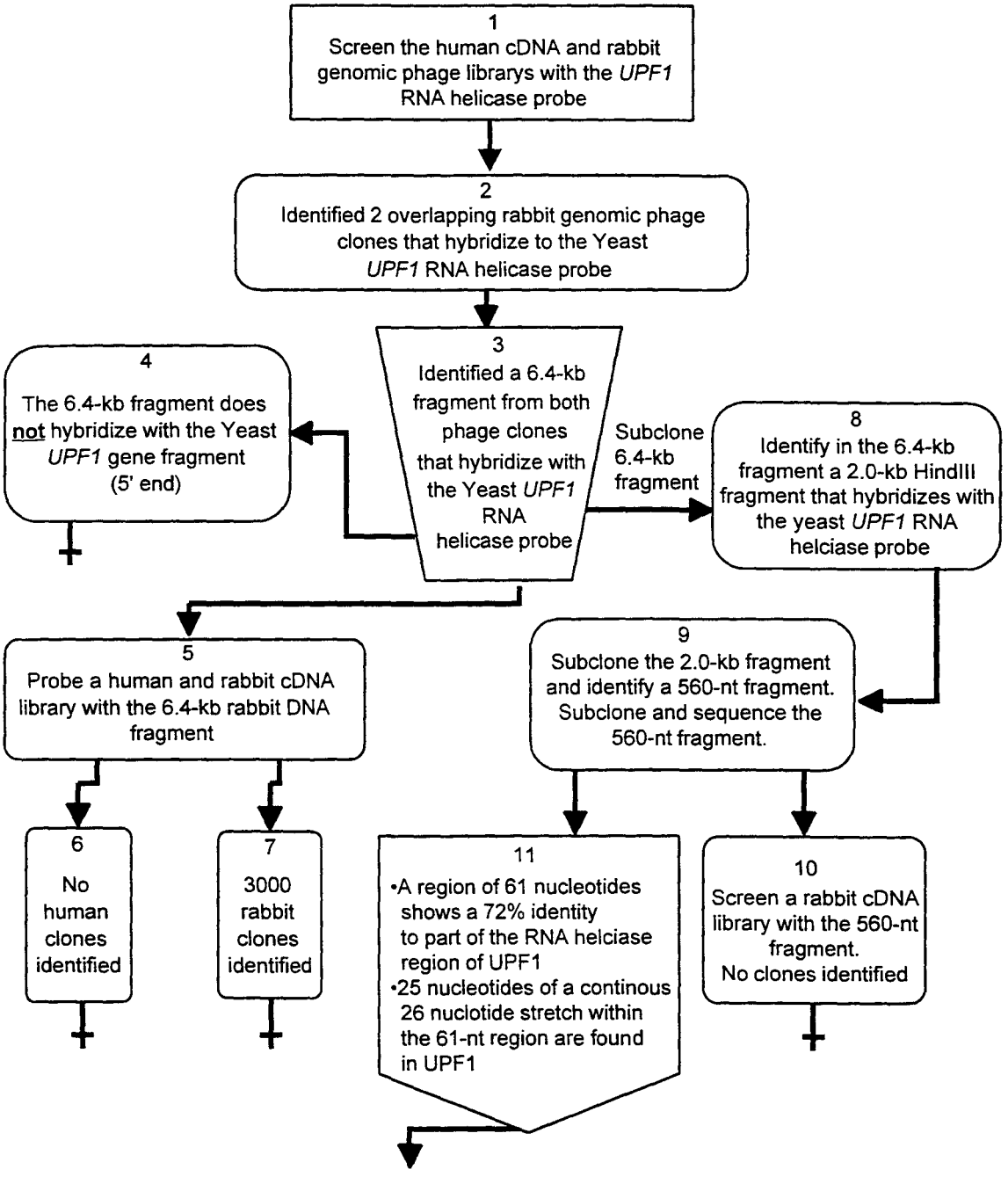


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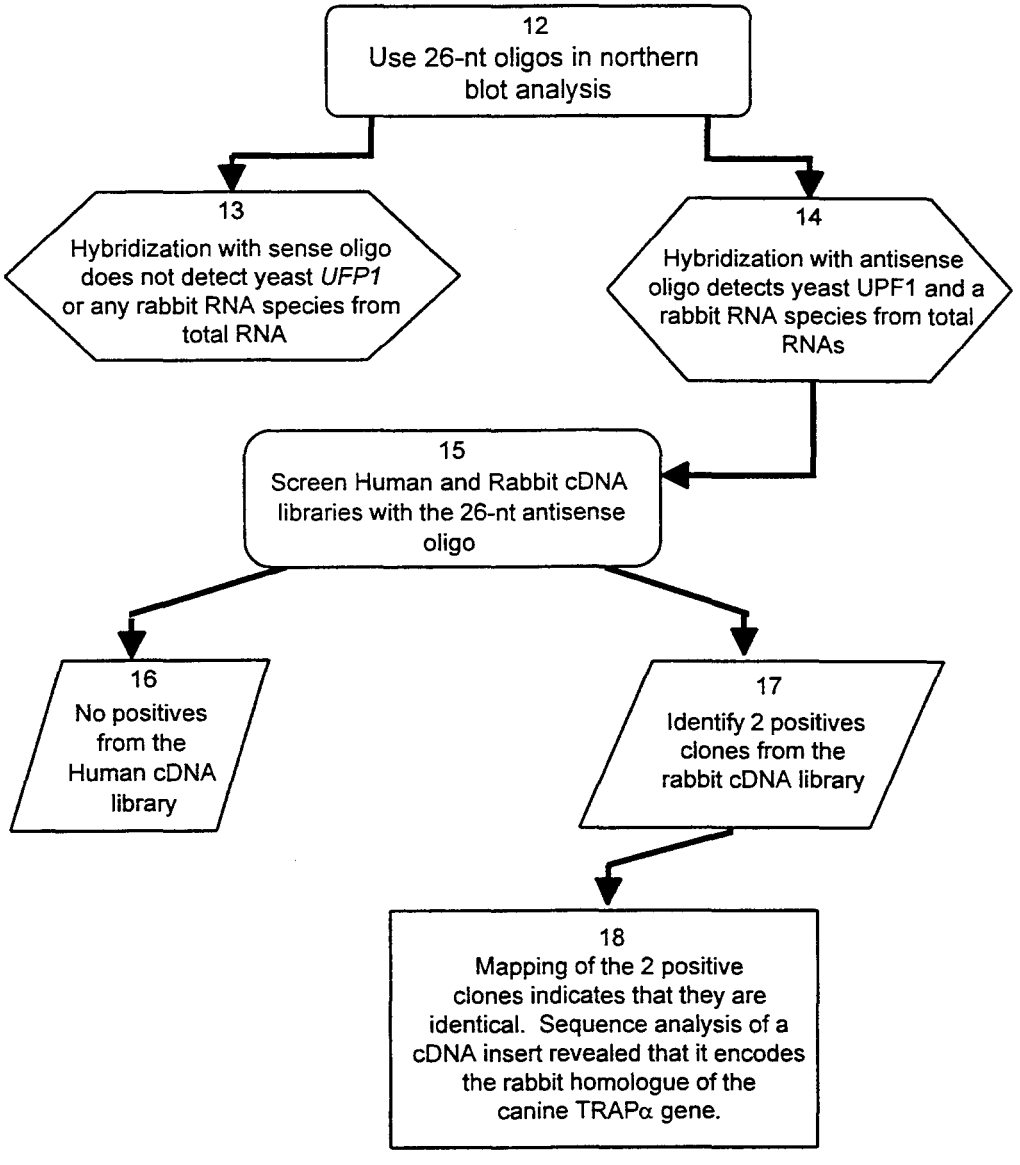
Figure 11. Flow chart representing steps taken during the isolation of a rabbit genomic clone that cross hybridizes with the yeast *UPF1* DNA probe and isolation of a rabbit cDNA.


12
Use 26-nt oligos in northern blot analysis
13
Hybridization with sense oligo does not detect yeast *UFP1* or any rabbit RNA species from total RNA
14
Hybridization with antisense oligo detects yeast UPF1 and a rabbit RNA species from total RNAs
15
Screen Human and Rabbit cDNA libraries with the 26-nt antisense oligo
16
No positives from the Human cDNA library
17
Identify 2 positives clones from the rabbit cDNA library
18
Mapping of the 2 positive clones indicates that they are identical. Sequence analysis of a cDNA insert revealed that it encodes the rabbit homologue of the canine TRAPα gene.

phage DNA and map the regions of the 2 clones. The results demonstrated that although the two rabbit genomic clones are slightly different, they contain DNA restriction sites at similar sites throughout their insert regions. I conclude that these two genomic phage clones are overlapping. I also carried out low stringency Southern blot analysis on the same restriction enzyme mapped DNA. I was able to detect a 6.4-kb *Eco*RI fragment in both clones which hybridized with the *UPF1* RNA helicase probe under low stringency conditions (Figure 11, box 3). I conclude that the 6.4-kb *Eco*RI fragment contains a region of nucleotide homology between the *UPF1* probe and the fragment of rabbit genomic DNA. To determine if the rabbit genomic fragment also contains nucleotide homology to the 5'-end of the yeast *UPF1* gene I used the 5' end of the *UPF1* gene as a probe on the same Southern blot using low stringency conditions. From this Southern I conclude that the insert region of the rabbit genomic phage clone does not specifically hybridize to the 5' end of the *UPF1* gene (Figure 11, box 4).

I used the 6.4-kb *Eco*RI fragment as a probe to isolate a cDNA fragment from a human or rabbit cDNA library (Figure 11, box 5). Unfortunately, I was not able to identify any human cDNA clones (Figure 11, box 6). However, by screening the rabbit cDNA library I was able to identify approximately 3000 hybridizing phage plaques (Figure 11, box 7). It is possible that the hybridization of most phage clones is due to regions within the 6.4-kb probe that have no homology to the yeast *UPF1* RNA helicase probe. To generate a smaller probe that hybridizes to the *UPF1* RNA helicase probe, I subcloned the 6.4-kb *Eco*RI

fragment into a plasmid vector, digested the vector with restriction enzymes and determined by Southern blot analyses which of the rabbit fragments hybridized to the *UPF1* RNA helicase probe (Figure 11, box 8) (data not shown). A 2.0-kb *Hind*III DNA fragment was identified, isolated and subcloned into a plasmid vector. Southern blot analysis of restriction digestion of the 2.0-kb *Hind*III fragment identified a 0.56-kb *Eco*RI/*Pst*I fragment that still hybridized with the *UPF1* RNA helicase probe (Figure 11, box 9). The *Eco*RI site comes from the vector and the *Pst*I site is located within the 2.0-kb *Hind*III fragment about 500-bp downstream of the vector SP6 site. I, therefore, predicted that if I sequence the plasmid that contains the 2.0-kb *Hind*III fragment with a SP6 primer, I would be able to find the region of nucleotide identity between the *UPF1* RNA helicase probe and the rabbit genomic DNA fragment. 832 nucleotides of the 2.0-kb insert were sequenced and confirmed the presence of a *Pst*I site 560-nt downstream of the SP6 site of the vector. When I searched the GenBank nucleic acid sequence database with the 832-nt rabbit sequence using the FASTA program (GCG Program Manual, 1994), I found that 72% of a 61-bp region (underlined position 399-460, Figure 12) of the 832-nt sequence are identical to the corresponding region in *UPF1* (Figure 13). Within the 61-nt region of identity, there is a 26-nt region of 96% identity to *UPF1* (position 399-424 of the rabbit genomic fragment, position 2051-2077 of *UPF1*, Figure 14) (Figure 11, box 11). Additional analysis of the rabbit sequence revealed the presence of a putative splice donor and splice

20h35ssr.Txt Length: 832 February 3, 1995

HindIII
AAGCTTATCGATTTCGAACCCGGGGTACCGAATTCTCCTATCCCATGCAT
GTGACTTCTAATATAATTATCAAATGTAGGACAGTGATACTGGACTTCAG
GTGTTACTTAAATCCCATCAGTTTTCCCACTTGTATTCTTTTTCAGACCC
AAGATCCTACACTGCGTTTGTCATTTCTCCCTGGTTTCCTGTAATCTGTG
AGAGTTCCTAGGCCTCCCAGTCACTCCCCGTCTTTTATGATCTTAGTACT
TTGACAGAATATTGGGCATTTTTTTTGGACCATGTCCTCAGGTTCTATTA
TGTCTTTTTATTTTTTTTGTGATTGAAGAGAATTTTTGCATTTGTGGCGA
GAAACAAATGATACAGTGTCCTTGGTGCAGGGTGTAAAGGGTTCTGTGAT
GTTGACATGTGTTGGTGCTGGTGACGTTAACTTTGATCACTTGGTTGAGG
TGTGAGGTTTGGACTCTAAAGCTGCTGCCCCTCCCTTTGCAGTTGGTAGA
TATCTTGGGGGCATGTTTTCAGTGCAAATATGACTCCTCTCACATGTTCA
TGTACTGATTTCAGCNTCGCTCGGTGGGGCTTGT**CTGCAG**CACTGAGGAG
TTCCCTTTCCCTTCATTACCCTTGTTGAGTGGAGTCTTCTCAAGGAGGAG
CTGTCCTTNNCCCGCATTTNTTTGCTTCTTAGCTCATCTCTGACTATGGA
TATGGATTCAAGGGTACTTGCATNTTTCTNTNNGTCCACACCTACTACTT
TCATGGTTTATTTNCTCAGATTGTGCAATTGTTGATCACGAGGAGCTCCT
TCAGTTTGCCTCCTGTTTTCTTTCTAAAAGCC

Figure 12. Partial nucleotide sequence of the 2.0-kb *Hind*III fragment of the genomic rabbit clone R5. The *Pst*I restriction site is indicated in bold and the *Hind*III cloning site is underlined. The region with nucleotide identity to the UPF1 probe is underlined. The identity was determined with a FASTA analysis using the 832-nt sequence.

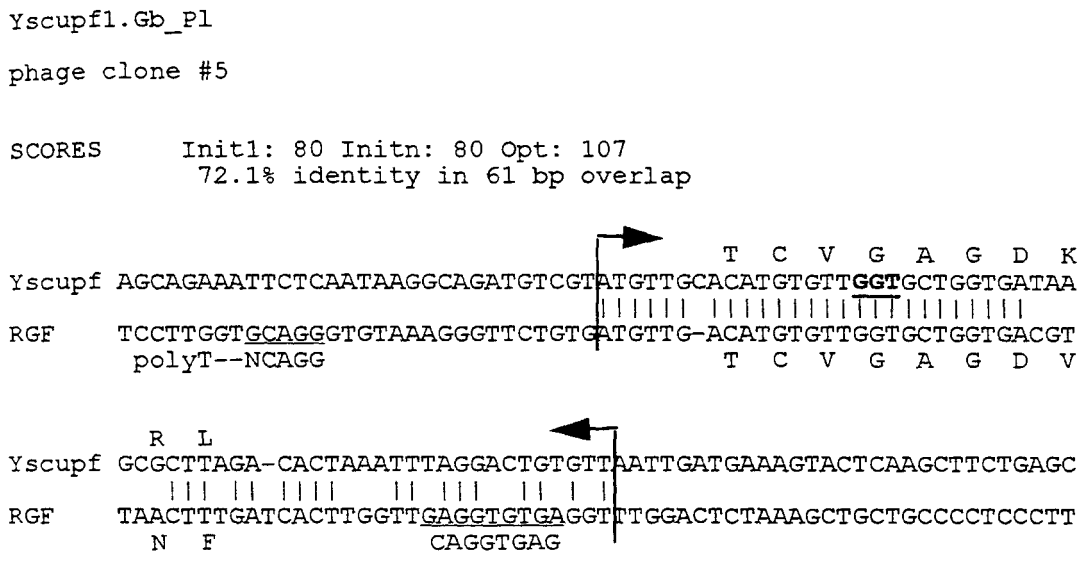


Figure 13. Original printout of the result of a FASTA analysis using 832 nucleotides of the 2.0-kb HindIII fragment of rabbit clone R5. Yscupf is part of the *S. cerevesiae UPF1* DNA sequence and RGF (rabbit genomic fragment) is part of the sequence of DNA shown in Figure 12. Underlined are the putative splice donor and acceptor sites found within the rabbit genomic fragment flanking the region of identity. The splice consensus sequences are shown below the underlined region (reviewed in Genes IV, Lewin, 1994). The putative amino acid sequence encoded by the rabbit genomic DNA and *UPF1* is shown above and below the respective nucleotide sequences. The aa changed in the D1 dominant- negative form of UPF1 is in bold and underlined. The region with 72% identity is shown between the arrows.

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20h3prim.For

Gb_Pl:Yscupf1

LOCUS       YSCUPF1      3546 bp ds-DNA        PLN       12-MAY-1992
DEFINITION  Saccharomyces cerevisiae zinc finger protein (UPF1) gene,
complete cds.

ACCESSION   M76659
KEYWORDS    UPF1 gene; zinc-finger protein.
SOURCE      Saccharomyces cerevisiae (strain GRF88) (library: Ycp50) DNA.

SCORES      Init1: 80 Initn: 80 Opt: 92
             96.3% identity in 27 bp overlap

                                                  10        20
20h3pr                                    ATGTTG-ACATGTGTTGGTGCTGGTGA
                                          |||||| ||||||||||||||||||||
Yscupf AGCAGAAATTCTCAATAAGGCAGATGTCGTATGTTGCACATGTGTTGGTGCTGGTGATAA
            2030      2040      2050      2060      2070      2080


Yscupf GCGCTTAGACACTAAATTTAGGACTGTGTTAATTGATGAAAGTACTCAAGCTTCTGAGCC
            2090      2100      2110      2120      2130      2140
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Figure 14. Origional printout of the result of a FASTA analysis using the 26-nt sequence (nt position 399-424 in Figure 12). Yscupf is the *S. cerevesiae UPF1* DNA sequence and 20h3pr is the sequence of the 26 bp oligonucleotide used to perform the analysis.

acceptor site (Lewin, Genes IV, 1995). Between the putative splice sites, one of the possible ribosomal reading frames could translate a putative protein encoding amino acids identical to those of *UPF1* (Figure 13). I conclude from these results that the cloned region of rabbit genomic DNA could contain an exon of a gene that is the rabbit homologue of the yeast *UPF1* gene, or a least a rabbit RNA helicase. Translation of an mRNA encoded by this region could lead to the production of a protein that has Upf1p like functions or RNA helicase activity.

Isolation of a Rabbit cDNA

To avoid extensive sequencing and exon/intron mapping of my genomic clone, I attempted to isolate a cDNA clone by screening appropriate libraries with a 26-nt oligomer that is identical to the 26-nt region (Figure 14). A 26-nt oligomer complimentary to the putative coding region of the rabbit genomic fragment was synthesized (26-nt antisense oligo) and used as a probe to determine if a RNA species can be detected in total spleenic RNA. When I performed northern blot analysis of total rabbit RNA from various tissues using the 26-nt antisense oligo as a probe, I detected a 1.3-kb band in total spleen RNA and a 3.6-kb band in yeast RNA with the 26-nt antisense oligo (Figure 11, box 14). The 3.5-kb band is very likely *UPF1* mRNA because a *UPF1* probe detects a band of the same size (data not shown). I screened 2.5 X 10^5 phage from a rabbit spleen cDNA library (a gift from Dr. Chander Raman) and from a human HeLa cDNA library with the

26-nt antisense oligo (Figure 11, box 15). I identified two rabbit cDNA clones that hybridized with the 26-nt antisense oligo (Figure 11, box 17) but no phage clones were identified from the human cDNA library (Figure 11, box 16). cDNA inserts from the two rabbit phage clones were rescued from the page particles by using PCR and a primer set (λgt10.fow/λgt10.bac) that flanks the cDNA insert. Agarose gel electrophoresis of the PCR product from each phage clone revealed a product of 1.2-kb (data not shown). I predicted that if the 1.2-kb PCR product actually contains regions of nucleotide identity with the *UPF1* gene it should be able to hybridize with a *UPF1* RNA helicase probe. The PCR products were, therefore, subjected to Southern blot analysis under low stringency conditions using the *UPF1* RNA helicase region probe. The results demonstrated that all PCR products as well as the original rabbit genomic phage clone are able to hybridize with the *UPF1* RNA helicase probe while a DNA fragment amplified by PCR from a rabbit GAPDH cDNA or λ-phage clones did not hybridize (data not shown). To determine whether the clones were identical, λ DNA was digested with various restriction enzymes. The results revealed the presence of identical restriction enzyme sites within both 1.2-kb inserts (Figure 11, box 18; data not shown). DNA sequencing of both clones was performed and revealed that their sequences are identical. The nt sequence of the 861 bp ORF of the two rabbit cDNA inserts differ by only one nucleotide (a wobble base leading to no aa change) and encodes for 286 aa (34.5-kDa). However, a poly(A)-addition signal sequence could not be located and comparison of the *TRAP*α sequence with the

putative RNA helicase region of *UPF1* revealed that TRAPα does not contain a helicase domain. FASTA and TFASTA analysis (GCG Program Manual, 1994) showed that the rabbit sequence has a high degree of nucleotide and amino acid identity to that of the canine translocon-associated protein alpha (*TRAP*α) gene (GenBank accession #X51367; Figure 11, box 18) (Figure 15) a gene product found to be associated with the translocon and associated proteins (Figure 16) (Hartmann et al., 1993).

The ORF has 89% and 94% identities to the canine *TRAP*α gene at the nt and aa level, respectively (Figure 15). The deduced aa sequences of both rabbit and canine TRAPα share conserved hydrophobic as well as acidic aa regions (Figure 15). Therefore, I conclude that I have isolated a full-length cDNA clone encoding rabbit *TRAP*α. Finally, part of the 26-nt antisense oligo sequence used to screen the rabbit cDNA library was located within the rabbit sequence (underlined and bolded in Figure 15), however, the sequence was translated in a reading frame that was different from that of yeast *UPF1* (compare aa sequence 250-255 in Figure 13 with aa sequence 551-555 in Figure 15). These results indicate that I have isolated a rabbit cDNA with a very high degree of nucleotide and amino acid identity to that of the canine *TRAP*α gene. Therefore, I conclude that I have cloned the rabbit homologue of the canine *TRAP*α gene whose gene product might be important for translocating nascent polypeptide chains from the cytoplasm across the ER membrane.

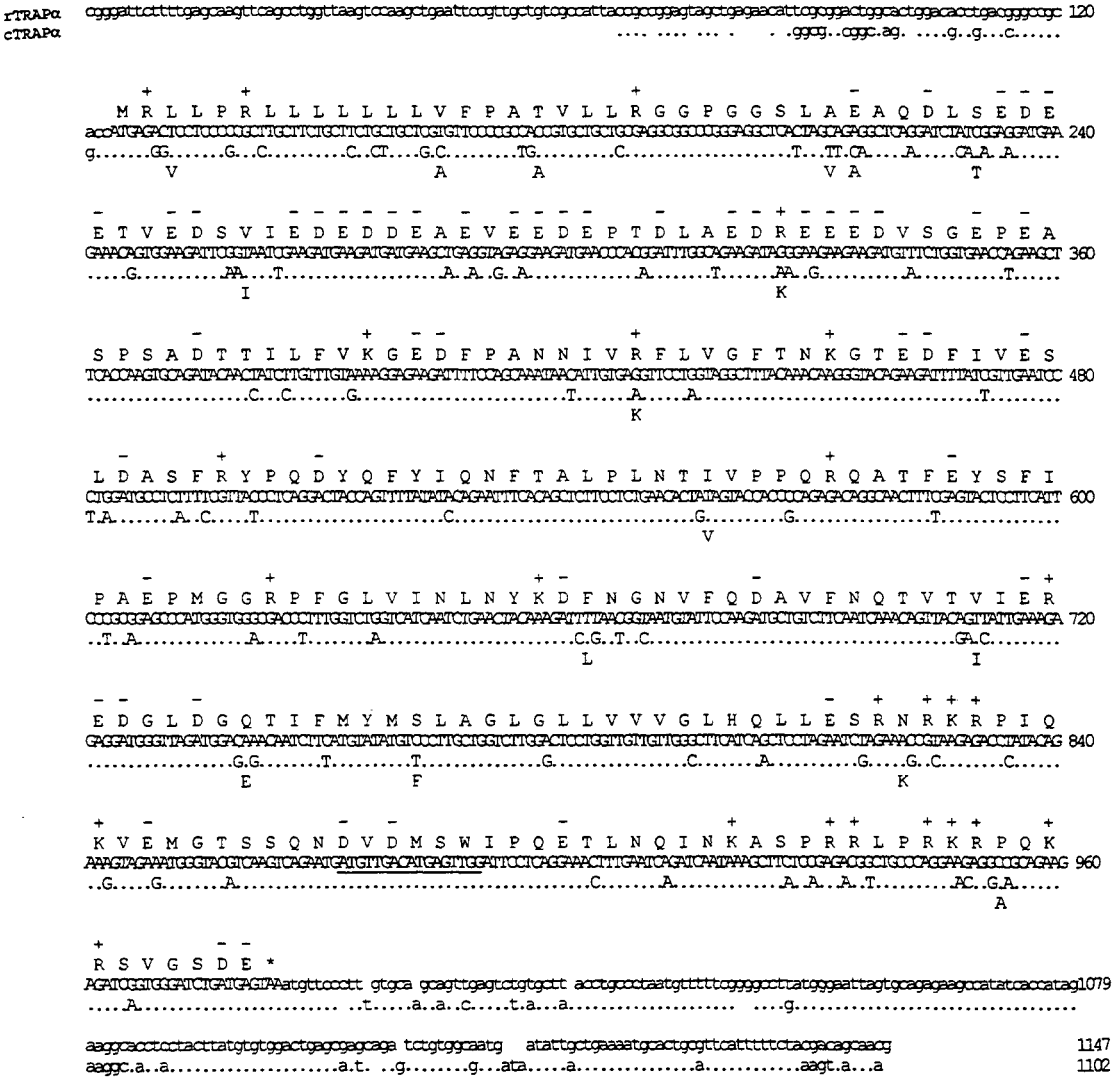


Figure 15. Comparison of nt and deduced aa sequences of rabbit and canine TRAPα. The nt sequence of rabbit TRAPα (rTRAPα) was determined as described (Sanger et al., 1977) from two cloned cDNA inserts. The nt sequence of canine TRAPα (cTRAPα) was retrieved from the GenBank database (accession no. X51367). In the alignments, a period indicates nt identity and a star a break in the nt sequence. The aa sequence predicted from the nt sequence of rTRAPα is shown above the codons in the single letter IUPC format, whereas only canine aa residues that are different from that of the rabbit aa sequence are shown below the canine nt sequence. The putative hydrophobic leader and transmembrane regions are underlined and the translational start and termination codons are double underlined. Acidic(-) and basic (+) aa residues are indicated and stretches of acidic aa residues, which are shared by rabbit and canine TRAPα.

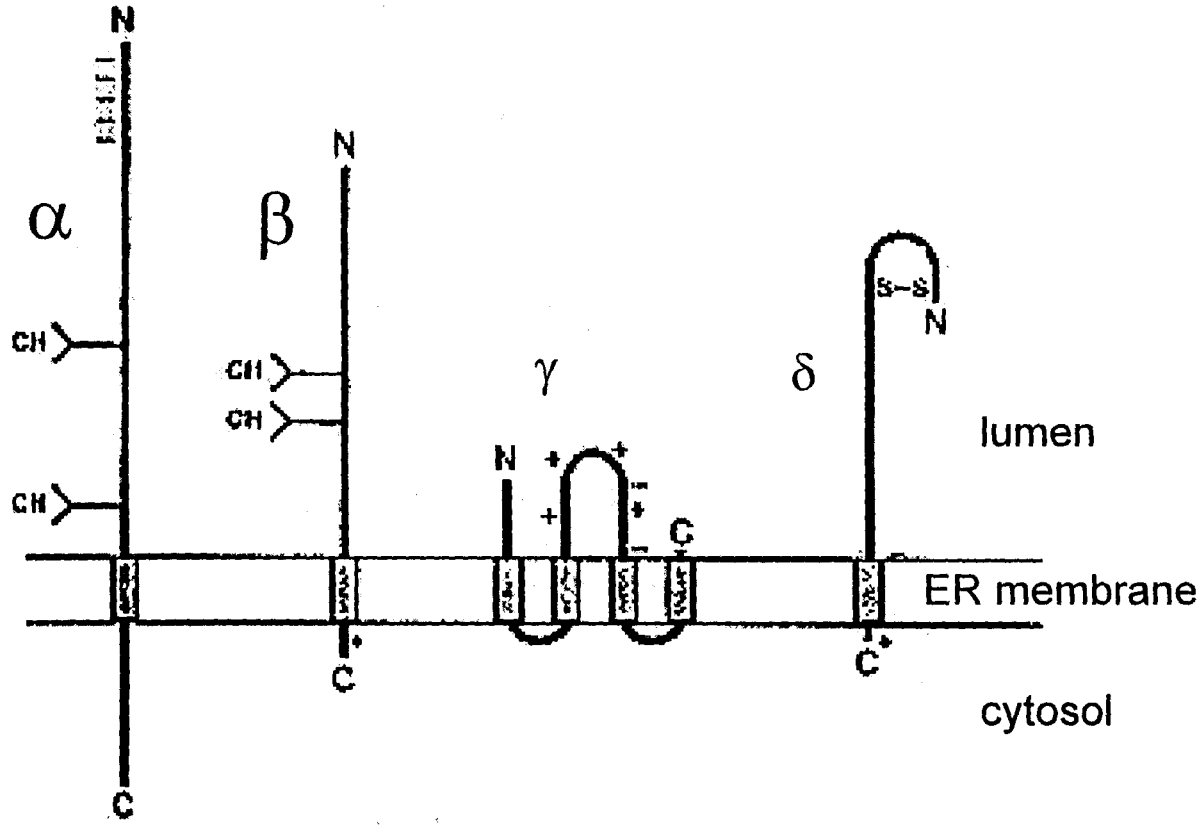


Figure 16. Schematic representation of the translocon proteins α, β, γ, and δ. Putative disulfide bonds, carbohydrate moities, and charged residues are indicated. Adapted from Hartmann et al., 1993.

Characterization of the Rabbit TRAPα cDNA

I carried out experiments in various mammalian cells to analyze the expression of *TRAPα* on the mRNA level in various mammalian cells (see Table 4). I analyzed total RNA from a rabbit (B cell hybridoma, 96-1), mouse (B cell lymphoma, NYC) and the human (T cell line, Jurkat) by northern blot analysis using high stringency conditions and the complete rabbit *TRAPα* cDNA clone as a probe. Figure 17.A shows the results from this experiment. In rabbit lanes I detected bands of 1.3, 2.8 and 3.7 kb (lane 2), in mouse 1.3, and 2.8 kb (lane 6), and in human lanes bands of 1.3, 3.7, and 2.7 kb in size (lane 4). From these results, I assume that the 1.3-kb band most probably represents *TRAPα* mRNA because its size corresponds very well with that of my rabbit *TRAPα* cDNA clone. Because I detect in the rabbit, human, and mouse cells very similar bands with the rabbit *TRAPα* probe, this gene is very likely conserved during evolution. This is further supported by the fact that *TRAPα* of other species such as human and trout (Hartmann and Prehn, 1994) are very similar to the canine and rabbit *TRAPα*.

To determine if the mRNA species larger than 1.3-kb are *TRAPα* mRNA precursors, I carried out northern blot analysis on the same cell lines using RNA isolated from the cytoplasm where only completely spliced *TRAPα* mRNAs should be present. Figure 17.A shows the results from this experiment. After probing the blot with the complete rabbit *TRAPα* gene, I was able to detect the

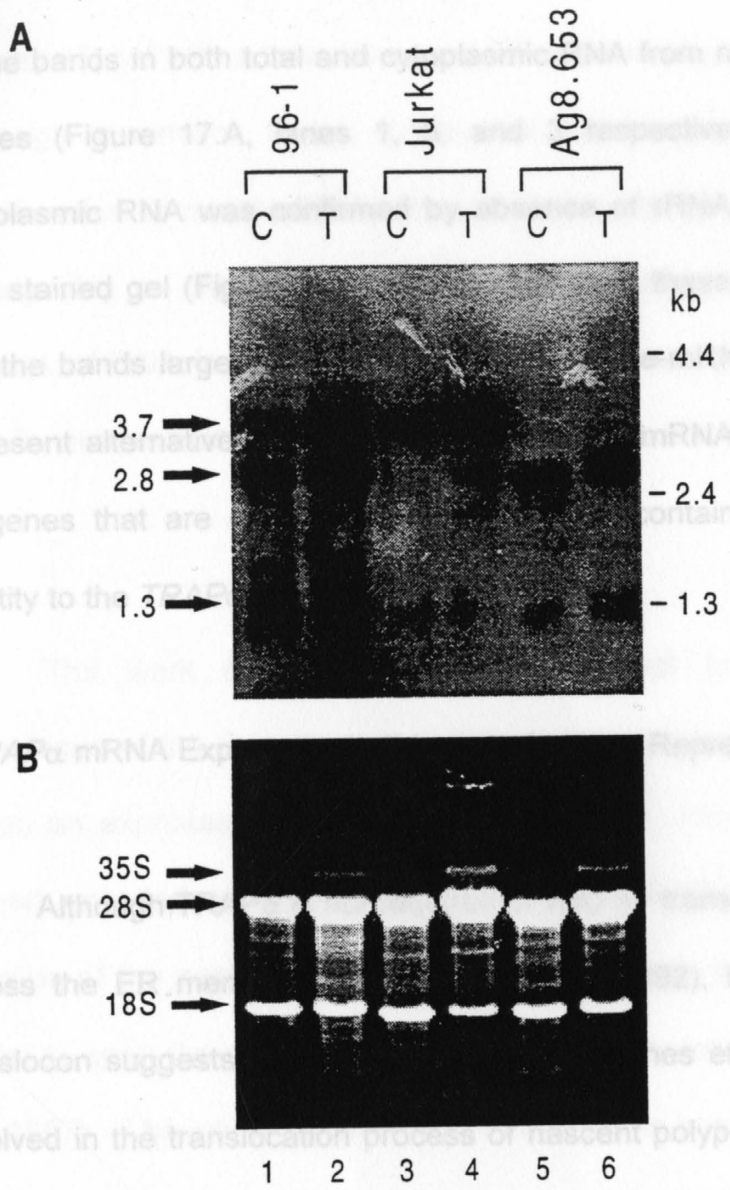


Figure 17. Northern blot analysis of total (T) and cytoplasmic (C) RNA from rabbit, human, and mouse cell lines. **(A)** Autoradiograph of a membrane probed with the complete ^{32}P-labeled rabbit TRAPα cDNA. **(B)** Photograph of ethidium bromide-stained gel. Total and cytoplasmic RNA were isolated from the rabbit B cell hybridoma, 96-1, the human T cell line, Jurkat, and the mouse plasmacytoma, Ag8.653. **(A)** 10μg RNA was separated in a denaturing 1.2% formaldehyde agarose gel and transferred to a nitrocellulose membrane. The filters were hybridized with the complete nick-translated ^{32}P-labeled rabbit TRAPα cDNA probe, washed under high stringent conditions and subjected to autoradiography for 3 days at -70^{0}C. **(B)** The absence of nuclear rRNA precursors above the 28S rRNA band (e.g., 35S and 45S) controls for purity of cytosolic, and the ratio of 28S:18S of about 3:1 for the integrity of the RNA.

same bands in both total and cytoplasmic RNA from rabbit, mouse and human tissues (Figure 17.A, lanes 1, 5, and 3 respectively). The purity of the cytoplasmic RNA was confirmed by absence of rRNA precursors (35S) in the EtBr stained gel (Figure 17.B). I conclude from these results that it is unlikely that the bands larger than 1.3-kb are *TRAP*α pre-mRNAs. Instead, they could represent alternatively spliced forms of *TRAP*α mRNAs or transcripts encoded by genes that are different from *TRAP*α but contain stretches of nucleotide identity to the *TRAP*α cDNA probe.

*TRAP*α mRNA Expression In B Lymphoid Lines Representing a B and Plasma Cell

Although TRAPα is not required *in vitro* for translocating nascent peptides across the ER membrane (Migliaccio et al., 1992), its close proximity to the translocon suggests a role in this process (Mothes et al., 1994). If TRAPα is involved in the translocation process of nascent polypeptide chains into the ER lumen, I would predict that TRAPα is expressed at higher levels in a cell with an extensive network of ER, which is characteristic for secretory cells. To test the idea, I analyzed *TRAP*α mRNA levels in antibody-producing cell lines that differ in their amount of ER and immunoglobulin secretion rate. I found that the antibody-secreting hybridoma cell NYCH, which represents a secretory plasma cell with an abundant network of ER (Jäck et al., 1992), expressed 2-3 X times more *TRAP*α mRNA (1.3-kb) than the B lymphoma line NYC, which represents

a mature B cell with a less developed ER network (Jäck et al., 1992) (Figure 18). Although it remains to be shown that TRAPα is required for polypeptide translocation across the ER membrane *in vivo*, our findings support the idea that it may play an important role in protein translocation. Whether the 2.8-kb species of RNA in mouse (2.8- and 3.7-kb in rabbit) play a role in producing TRAPα protein that aids in protein translocation is unknown.

Conclusion

The work above describes my attempt to clone the mammalian homologue of the *S. cerevesiae UPF1* gene using low-stringency hybridization. When an expressed gene (cDNA) was finally isolated, I found that the rabbit cloned encoded a homologue of the canine *TRAPα* (Figure 15). The exact function of the canine *TRAPα* gene is not known. It is known to be a protein found to span the membrane of the endoplasmic reticulum (ER) (Wiedmann et al., 1987). Though its function is not known, it is possible (as deduced from its location) that it is involved with the process of protein translocation across the ER membrane (Prehn et al., 1990). It is in close proximity with three other proteins called TRAP β, γ and δ and are thought to be part of the translocon (Hartmann et al., 1993), a group of proteins found at the site of protein translocation. So far there is evidence for and against the participation of TRAPα in the translocation of proteins with leader sequences across the

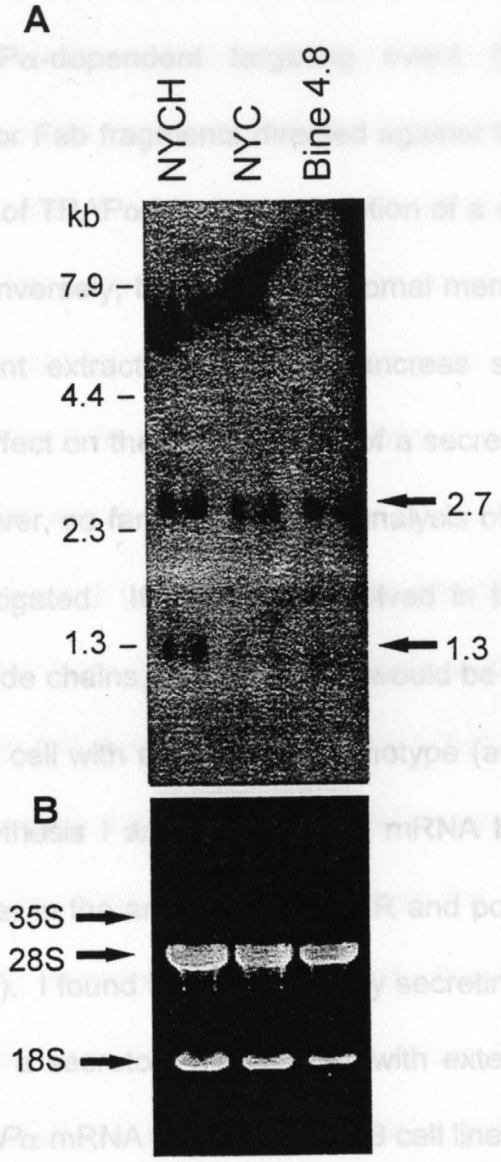


Figure 18. Northern blot analysis of various mouse B lymphoid cell lines. **(A)** Autoradiograph of a membrane probed with a complete, radiolabeled rabbit TRAPα cDNA probe. **(B)** Photograph of ethidium bromide-stained gel. 10μg of total RNA was separated and analyzed as described, except that the blot was washed under low stringent conditions (2xSSC / 0.1%SDS / 55^{0}C, 2 x 15 min). The membrane was subjected to autoradiography for 5 days at -70^{0}C. Signal levels associated with the 1.3-kb TRAPα band were determined by densitomitry and normalized to the amount of radioactivity determined for the 2.7-kb cross-hybridizing band.

membrane of the ER. Cross linking experiments have identified TRAPα as a protein residing in the vicinity of the signal sequence of a polypeptide chain during the TRAPα-dependent targeting event (Wiedmann et al., 1987). Additionally, IgG or Fab fragments directed against the cytoplasmically exposed carboxy terminus of TRAPα block translocation of a secretory protein (Hartmann et al., 1989). Conversely, by using microsomal membranes made from TRAPα depleted detergent extracts of canine pancreas show that the depletion of TRAPα had no effect on the translocation of a secretory precursor (Migliaccio et al., 1992). However, so far no molecular analysis of *TRAPα* at the mRNA level have been investigated. If TRAPα is involved in the translocation process of nascent polypeptide chains, one prediction would be that TRAPα is expressed at higher levels in a cell with a secretory phenotype (an extensive network of ER). To test this hypothesis I analyzed *TRAPα* mRNA levels in antibody-producing cell lines that differ in the amount of their ER and potential to secrete antibodies (Jäck et al., 1989). I found that the antibody secreting B cell hybridoma, (NYCH) which represents a secretory plasma cell with extensive ER produces 5 times more 1.3-kb *TRAPα* mRNA than its parent B cell line (NYC) (Figure 18).

Although it remains to be shown that TRAPα is directly required for polypeptide translocation across the ER membrane *in vivo*, my findings support the idea that it may be important in protein translocation. The above conclusion is, however, based on mRNA expression analysis. First of all, the increase in 1.3-kb mRNA levels have not been confirmed on the protein level using an

antibody against the TRAPα protein. Second, although there was a 5 fold increase in 1.3-kb mRNA level, there was no difference in 2.8-kb mRNA level. It appears that this mRNA is not under the same type of control as that of the 1.3-kb species. This could be due to differential transcription rate or to different mRNA stability between the two cell types. Finally, it is not clear whether TRAPα protein is indeed transcribed from the 1.3-kb transcript. The 1.3-kb transcript could also be encoded by a cross-hybridizing gene. In this case, TRAPα would be encoded for by the 2.8-kb or 3.4-kb transcript.

As mentioned above there is evidence for and against TRAPα's role in the translocation of nascent polypeptide chains into the lumen of the ER. The evidence described here indicates that when a cell secretes more proteins (immunoglobulin in this case) it makes more 1.3-kb *TRAPα* mRNA. Based on the assumption that the 1.3-kb *TRAPα* mRNA encodes the TRAPα protein, it is tempting to speculate that as a cell increases the size of its ER (and thus its secretion potential), it increases TRAPα production to facilitate the translocation process of secreted proteins. As mentioned above, TRAPα may not be essential for protein translocation. However, it could, together with essential proteins, serve as a translocation enhancer.

Transfect an *upf1* Null Yeast Strain With a Mammalian cDNA Expression Library and Rescue Yeast Transformants With a UPF1-Positive Phenotype.

Mammalian homologues of the yeast *S. cerevisiae* genes can be isolated by complementation of a mutant yeast strain with a candidate mammalian gene or a mammalian expression library. This process has a number of steps. First, the identification or generation of a strain that doesnt express the gene of interest (mutant allele) and changes its phenotype, for which one can select, depending on the presence or absence of the gene of interest. Second, the mutant yeast is then transformed with a cDNA library generated from the organism from which the homologue will be cloned. Third, yeast transformants are selected, or screened, for a positive phenotype. Fourth, the introduced gene is isolated from the yeast and analyzed for structural (sequence) and functional homology (antisense expression in mammalian cells). This approach is only successful if the mammalian gene product replaces the function of the absent yeast protein.

The first goal was to generate a *UPF1* mutant yeast strain (*upf1*) that changes its phenotype depending on the presence or absence of Upf1p, e.g. doesn't grow in the absence of an essential nutrient. For example, when the *HIS4* gene contains a nonsense codon the mRNA is usually degraded and the yeast is unable to grow in the absence of histidine (Leeds et al., 1992), however, when the *UPF1* gene is absent from this yeast cell the mRNA becomes stable leading to an increase in *his4* mRNA levels and, due to a subsequent ribosomal read-through of the nonsense codon, an increase in the level of His4p. Although

this selection scheme is useful to isolate *upf1* mutant yeast strains, it might be difficult to use it for isolating a mammalian homologue. This is because the complementation of a *upf1* mutant would render a His^+ cell His^-, which would require the replica-plating of thousands of transformants on His^- and His^+ plates. The selection system I have devised uses negative selection marker in *S. cerevisiae*, the *CAN1* gene product. The *CAN1* gene encodes arginine permase, a outer transmembrane protein that transports the amino acid arginine. The *CAN1* gene product can also import the toxic arginine analogue canavanine. Canavanine is able to act as a chain terminator during protein translation and leads to the death of the yeast cell. As expected, cells that have a functional *CAN1* gene are sensitive to medium containing canavanine and cannot grow whereas *can1* yeast (Can^R) are able to grow in canavanine (reviewed by Rosenthal, 1977). My selection system employs the *CAN1* gene to select for *upf1* mutant yeast cells that have been complemented with a mammalian *UPF1* homologue. I proposed to generate a yeast strain containing a *can1* gene with a nonsense codon (canavanine resistance) and a defective *UPF1* gene. If the loss of *UPF1* leads to an increase in nonsense *can1* mRNA and if there is enough translation read-through, more Can1p should be synthesized, leading to a canavanine sensitive phenotype. Or in other words, the yeast cells should not grow in the presence of canavanine (Figure 19). The complementation of this sensitive strain with a mammalian *UPF1* homologue would then lead to the

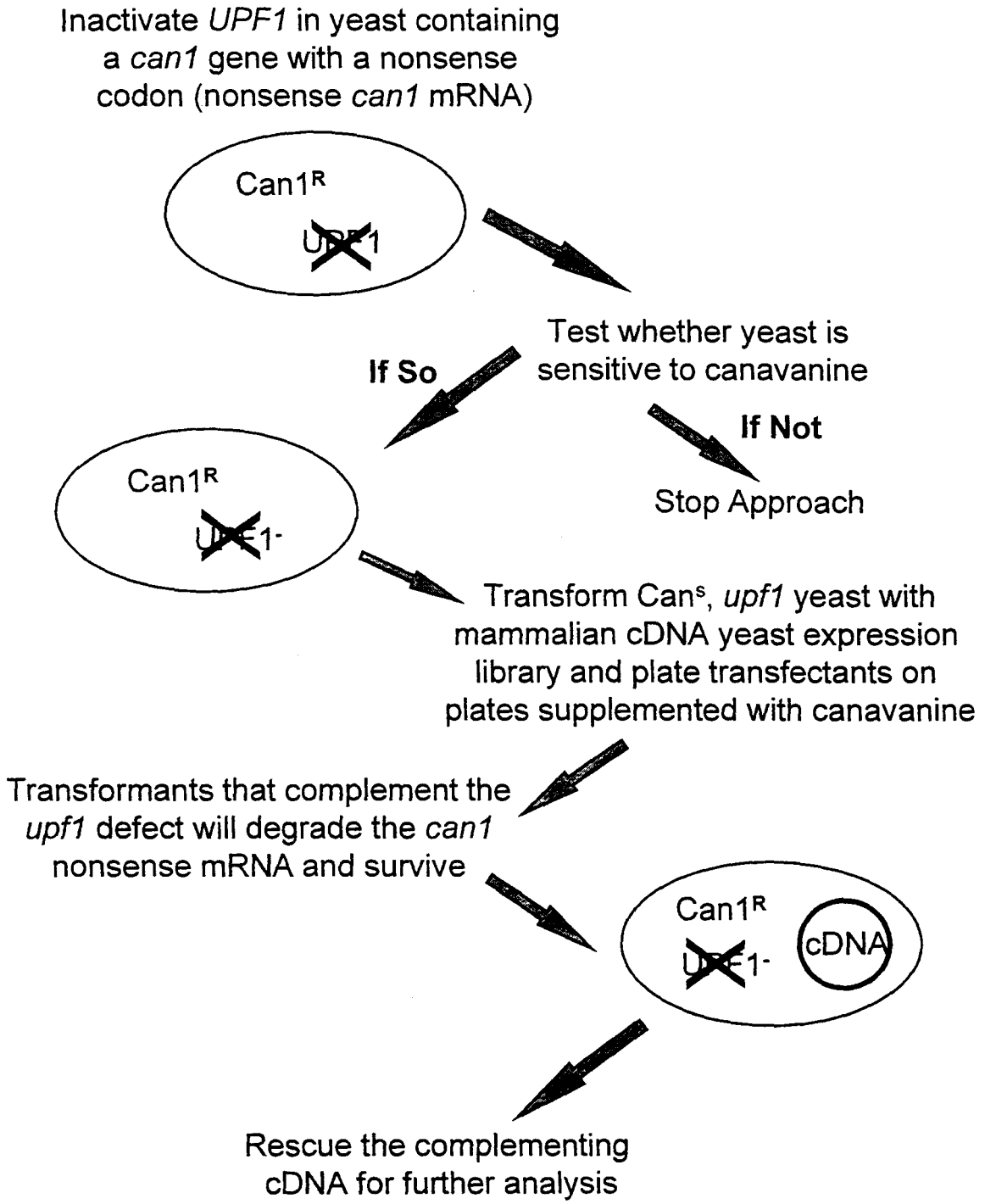


Figure 19. Schematic diagram of the complementation approach to isolate a mammalian *UPF1* homologue by complementation of a UPF1$^-$ yeast strain. Can1^R, grows in canavanine, Can^S, doesn't grow in canavanine, UPF1$^-$, UPF1 is deleted.

degradation of the nonsense *can1* mRNA and thus, to decreased Can1p levels. Consequently, yeast cells that have been complemented with a functional *UPF1* homologue, should grow in the presence of canavanine (Figure 19). In this way only yeast receiving a mammalian homologue of the *UPF1* gene would grow and these cells could easily be isolated for study. Because I was unable to generate a suitable *upf1*, *can1* nonsense, yeast strain, I will only briefly discuss this approach in the following sections.

Creation of a Selectable Yeast Strain That Changes Its CAN1 Phenotype Depending on the Presence of Upf1p

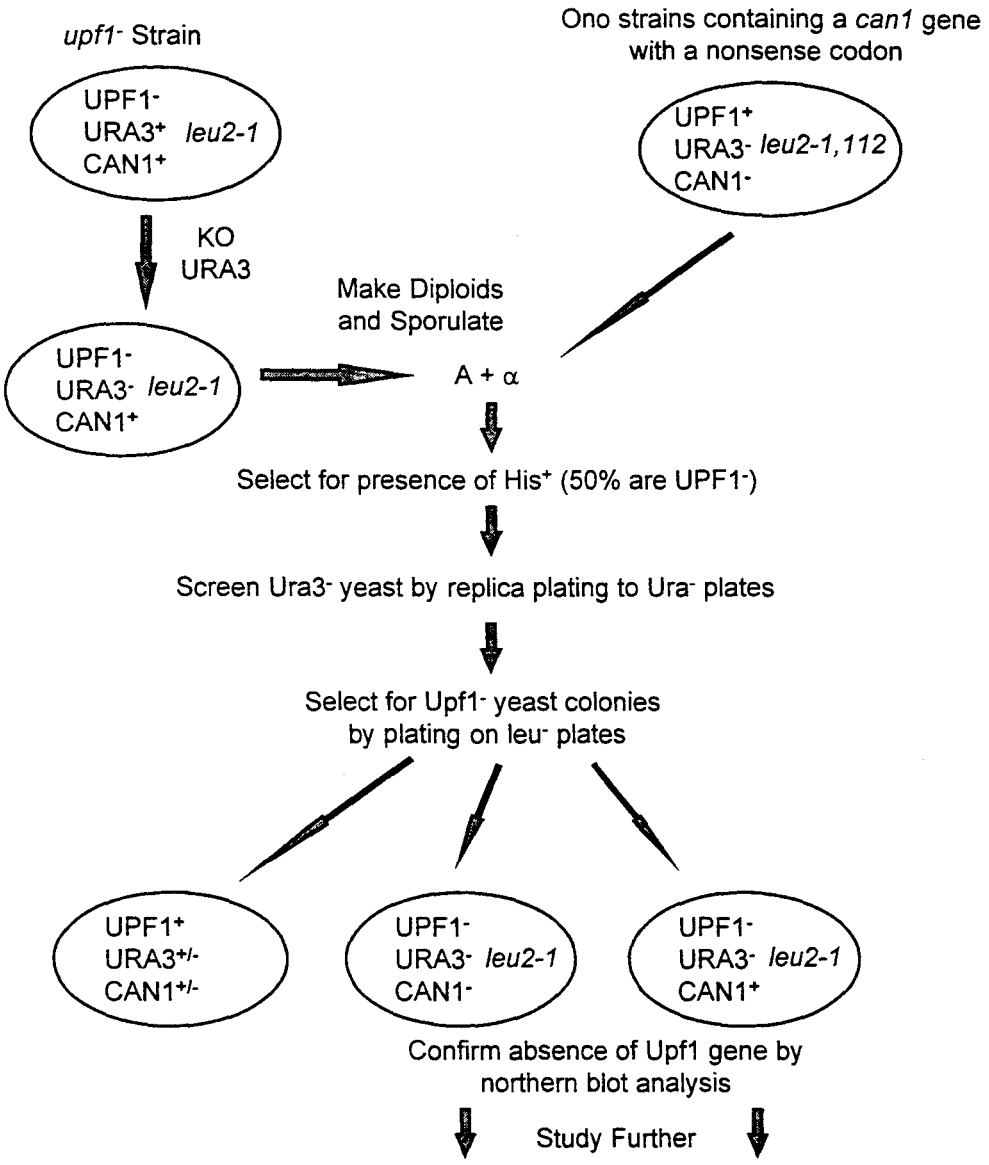
To carry out this approach I obtained yeast strains that contain *can1* genes with different nonsense codons (Ono et al., 1983) and a *UPF1* mutant strain (Table 6) (Leeds et al., 1991). These strains were tested and have the correct phenotypes (data not shown). One important part of the *UPF1* positive selection system is that the levels of *can1* nonsense mRNAs are decreased in the presence of the Upf1p. Because at the beginning of these experiments I didn't have an antibody against the Upf1p, I performed northern blot analysis and found that nonsense *can1* mRNA levels in the three strains Ono 279, 280, and 284, were approximately 50% lower when compared to the steady state level of functional *CAN1* mRNA in the parent strain Ono26 (data not shown). From these results I conclude that the presence of an *UPF1* mRNA correlates

Table 6. Yeast Strains

Strain	Genotype	Source
Ono 26	*MATa leu2-1,112 met8-1*	B.I. Ono
Ono 280	*MATa leu2-1,112 can1-204 met8-1*	B.I. Ono
Ono 284	*MATa leu2-1,112 can1-202 met8-1*	B.I. Ono
PLY154-*ura3::his4*	*MATα upf1-D1 ura3-52 trp1-D1 rpb1-1 his4-38 leu2-1 ura3::HIS4*	This work
PLY154	*MATα upf1-Δ1 ura3-52 trp1-Δ1 rpb1-1 his4-38 leu2-1*	M. Culbertson
YO6-15C	*MATa ade2-1 aro7-1 can1-100 leu2-2 ilv1-2 his4-166 lys2-101 met8-1*	B.I. Ono
SL988-11A	*MATα leu2-1 lys2-1 his3-Δ1 ura3-52 trp1*	S. Liebman

with a decrease in the steady state level of *can1* mRNA containing a nonsense codon when compared to the parent strain. This result raises the possibility that upon elimination of the *UPF1* gene in the *can1* nonsense codon yeast strains, the level of *can1* nonsense mRNA will increase and that this increase combined with possible ribosomal read-through of the nonsense codon will lead to the production of enough Can1p to change the phenotype of the *UPF1* mutant strain from canavanine resistant to canavanine sensitive.

The following briefly describes the steps involved in the generation of a suitable yeast strain that can be used to select for the presence of a mammalian *UPF1* homologue (Figure 20). First, I inactivated the *URA3* gene by insertion of a selectable marker. This will allow selection of transformants containing URA3



Test *upf1* (Upf1-), *ura3* (Ura3-) yeast for canavanine sensitive. If yeast are only canavanine sensitive, it indicates that the absence of *UPF1* may be de-suppressing the *can1* nonsense mRNA leading to the production of CAN1 protein and thus, canavanine sensitivity. Confirm this effect by *UPF1* transformation and complementation. If both canavanine sensitive and canavanine resistant yeast exist it indicates that the absence of *upf1* cannot de-suppress the *can1* nonsense mRNA and the yeast cannot be used to identify a *UPF1* mammalian homologue by complementation.

Figure 20. Flow chart to generate a suitable yeast strain to isolate a mammalian UPF1 homolgue by complementation.

plasmids (data not shown). To generate a *upf1*, *ura3*, nonsense *can1* strain which will finally be used as the recipient for the mammalian cDNA library, I used a mating and sporulation approach. To do this the appropriate yeast strains (PLY154 *ura3::HIS4*, with Ono 26, 280, 284) were mated, sporulated, and selected for the presence or absence of appropriate markers according to the protocol described in materials and methods. To select for the presence of the *upf1* gene disrupted by an insertion of the *HIS4* gene, the haploid segregates were screened for the ability to grow in His^- media. Because the *can1* nonsense codon strains (Ono 26, 280, 284) all contain a wild-type *HIS4* gene, I expected that only 50% of the His^+ yeast would contain a disrupted *UPF1* gene. To identify the yeast with a disrupted *UPF1* gene I replica-plated the His^+ yeast colonies on plates without leucine. Because the haploid segregates contain either a *UPF1*-suppressable (*leu2-1*) or *UPF1*-non-suppressable (*leu2-1,112*) allele of the *leu2* gene, only cells that do not have *UPF1* and contain the suppressible allele of *leu2* can grow in the absence of leucine. The Leu^+ yeast colonies were then replica plated onto Ura^- plates to obtain yeast that are auxotrophic for the library selection marker (*URA3*). To confirm the absence of a functional *UPF1* gene, RNA from these yeast colonies was subjected to northern blot analysis using a *UPF1* probe (Figure 21). The results demonstrate that the Ura^-, His^+, Leu^+ yeast colonies do not express *UPF1* mRNA when compared to the parent Ono strains. These yeast colonies will contain either a wild-type or nonsense codon *CAN1* allele (to be discussed below) (Figure 20).

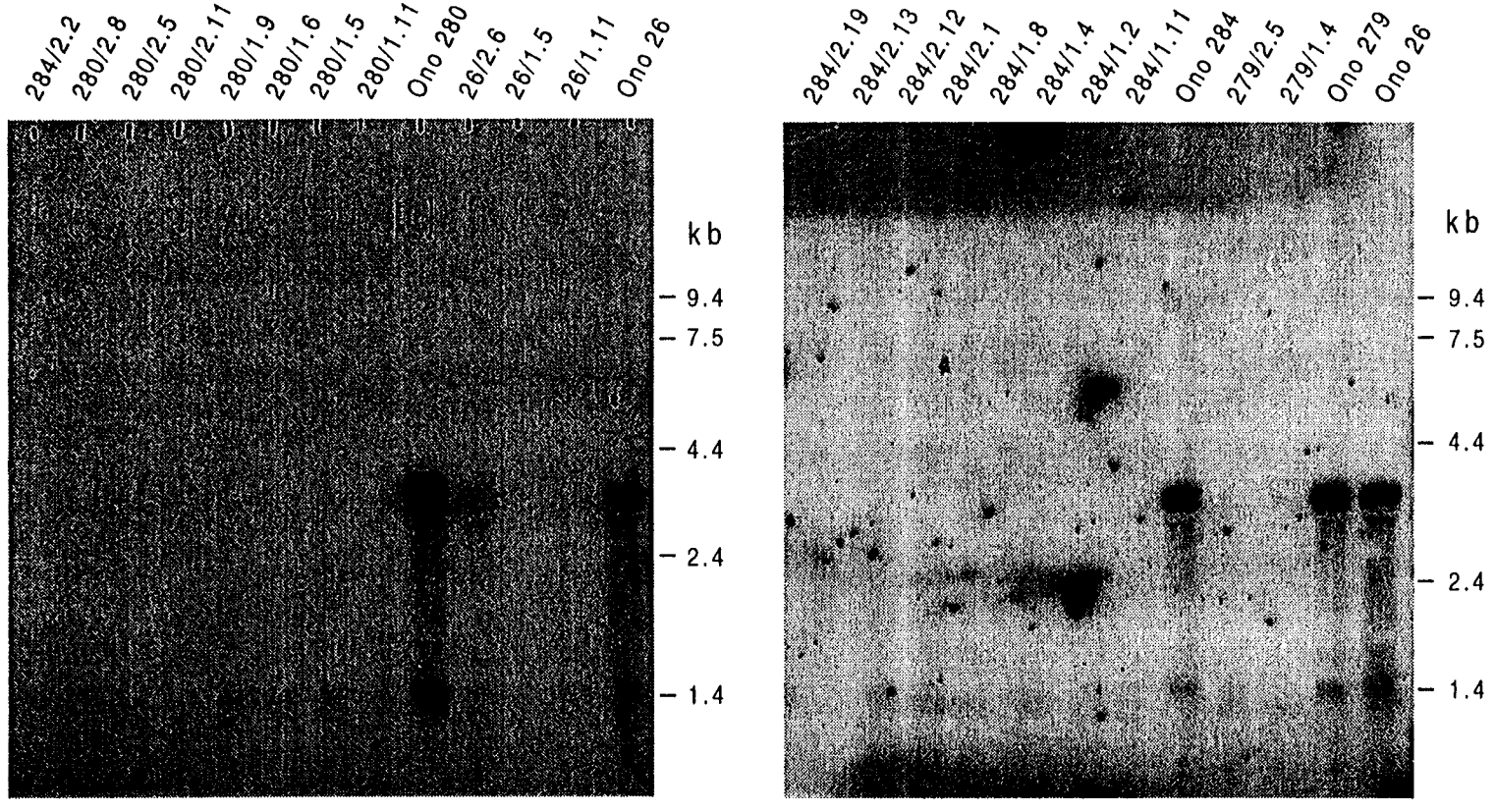


Figure 21. Northern blot of total yeast RNA using a *UPF1* probe. 5 µg of total RNA from HIS$^+$, URA$^+$, LEU$^+$ yeast colonies was subjected to northern blot analysis using a complete *UPF1* probe. Filters were exposed for 2 days at -70°C using one enhancing screen. Ono 279, 280, 284 contain a nonsense *can1* mRNA and Ono 26 contains a functional *CAN1* gene and serves as a positive control for hybridization. Sizes (in kb) of molecular size markers are indicated on the right of the blots. EtBr staining (not shown) verified that about the same amount of RNA was loaded in all lanes.

To test whether the *upf1* mutant yeast is able to suppress a *can1* nonsense codon allele and change the phenotype of the yeast from canavanine resistant to canavanine sensitive, I determined the sensitivity of the yeast colonies to canavanine. Briefly, yeast were grown overnight in liquid cultures and counted according to the optical density. Ten-fold dilutions of cells were spotted onto plates containing increasing levels of canavanine from 0 μg/ml to 150 μg/ml. All the parent strains used to create the new haploid crosses were also plated on these plates to control for a wild type *CAN1* or a nonsense *can1* gene. Each cross of the *upf1* null strain with Ono 280 as well as Ono 284, both of which contain nonsense *can1* genes, yielded colonies that are completely resistant to canavanine, or that are completely sensitive to canavanine (Figure 22). This suggests that all yeast colonies received a *CAN1* allele from either the *upf1* parent (PLY154 *ura3::HIS4*, *CAN1*), or from the Ono strains (Ono nonsense strains, *can1*). Further, I conclude that the absence of Upf1p does not lead to the suppression of the *can1* nonsense codon mRNA in Ono 280 and Ono284. Thus, none of the crossed yeast strains, although some of them contain a nonsense *can1*, and each of them *upf1*, are suitable for the selection of a functional *UPF1* gene or mammalian homologue.

Conclusion

I have shown here that the presence of a *UPF1* gene correlates with the

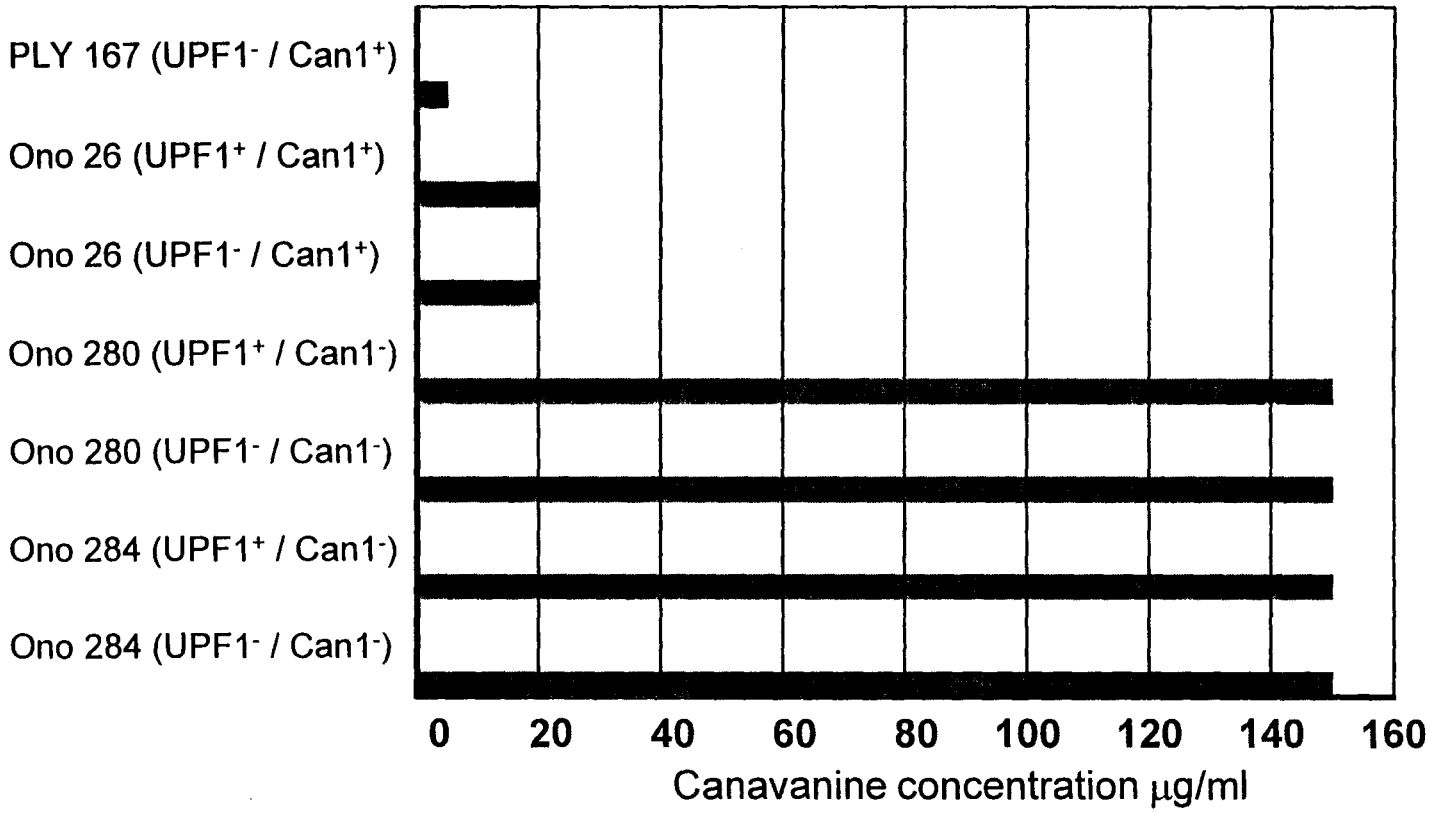


Figure 22. Growth of various yeast strains on canavanine . Bars represent the degree of growth of various mutant $UPF1^{+/-}$, $CAN1^{+/-}$ yeast strains on canavanine plates.

decreased steady state level of a *can1* mRNA containing a nonsense codon. Unfortunately when the *UPF1* gene is absent, the phenotype of the mutant yeast cells does not change from canavanine resistant to canavanine sensitive. Because this change does not occur, it is not possible to use these yeast strains as recipients for a mammalian cDNA library to select for cDNA clones that complement the *upf1* defect. Two explanations might be that levels of *can1* nonsense mRNA do not significantly increase in *upf1* cells or that an increase in Can1p is not enough to confer canavanine sensitivity.

Determine Whether the Expression of a Dominant-Negative Form of Yeast Upf1p In a Mammalian Cell Correlates With Increased Levels of Nonsense mRNA

Overall Approach and Rational

The use of dominant-negative genetic systems has become widespread in identifying the function of a gene in lower eukaryotic and mammalian organisms. This technique involves the expression of a truncated or mutant form of a gene whose product can interfere with the normal function of its corresponding wild type gene. The mutant gene is thought to interfere by competing for interacting molecules, thereby inhibiting the ability of the wild type gene product to carry out its intended function, or by interacting directly with the wild type gene product and, thereby, eliminating its function. Alternatively, a truncated or wild-type polypeptide could gain a function, which interferes with other physiological processes within a cell.

It is possible that dominant-negative forms of Upf1p (Upf1-D1p), which have been identified in yeast cells, could also act dominant-negative in a higher eukaryotic cell. If mammalian cells contain a *UPF1*-Like protein, I expect that the expression of yeast Upf1-D1p in a mammalian cell could result in increased levels of nonsense mRNA (Figure 23). If this is the case, it would be the first evidence that a *UPF*-Like system exists in mammalian cells.

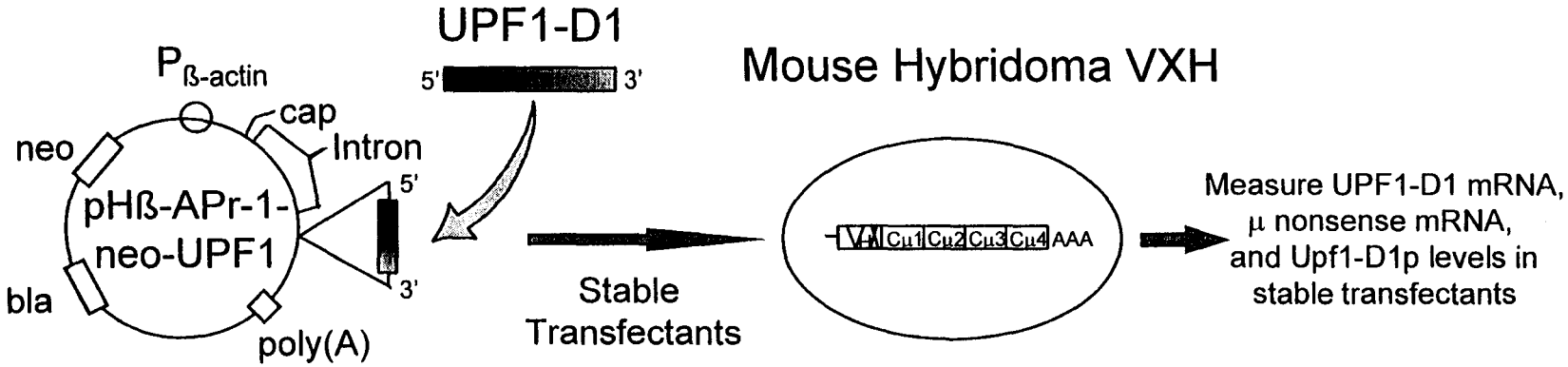


Figure 23. Strategy to identify a UPF1-like system in a mammalian cell by expression of a dominant-negative form of UPF1. The Schematic representation of the cloning of *UPF1-D1* into the expression vector pHβ-APr-1-neo and subsequent transfection into the mouse B cell hybridoma VXH. Structure of the nonsense μ mRNA in VXH is shown in the cell. The exons are indicated by boxes (V_H variable; C, constant) and the length of translation products by lines below the mRNA. The position of the nonsense codon at the end of the VH exon is represented by the X.

Generation of a Mammalian UPF1-D1 Expression Vector

To determine if a dominant-negative form of the yeast Upf1p can block the function of a putative mammalian factor that controls nonsense mRNA levels, I obtained a dominant-negative *UPF1* mutant gene. This gene called *UPF1-D1*, contains a missense mutation within the RNA helicase region (Gly556Asp). Wild type yeast transformed with this mutant *UPF1-D1* behave as if they lack the *UPF1* gene, that is, they grow in the absence of leucine, although they still contain a nonsense *leu2-1* mRNA (see page 34 for explanation). From these results it was determined that this *UPF1* mutant acts in a dominant-negative fashion on the wild type *UPF1* expressed within the same cell (Leeds et al., 1992). To carry out this approach, I cloned the *UPF1* and *UPF1-D1* genes into a mammalian expression vector as described in Figure 24. To confirm that the *UPF1-D1* construct was not altered during the cloning procedure, I confirmed the D1 mutation by DNA sequencing and performed western blot analysis on *in vitro* transcribed-translated products generated from an *UPF1-D1* plasmid. I was able to detect the D1 mutation after the swapping of the D1 fragment (Figure 25) and a radiolabeled band of the same size produced from *in vitro* transcribed-translated *UPF1* and *UPF1-D1* plasmids (Figure 26). This indicates that the cloning of *UPF1* and *UPF1-D1* did not alter the ORF.

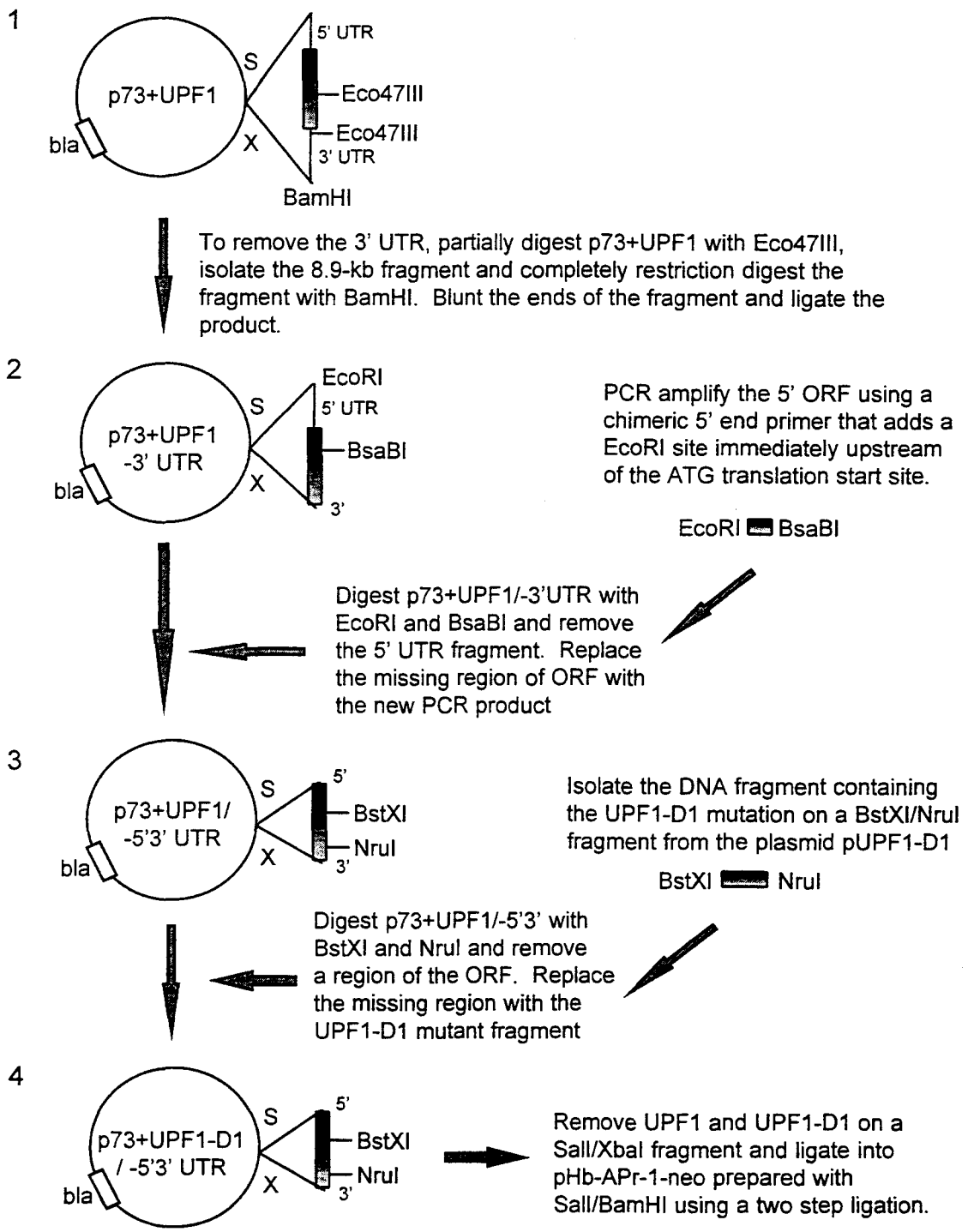


Figure 24. Schematic representation of the steps taken to clone *UPF1* and *UPF-D1* ORF into the mammalian expression vector pHβ-APr-1-neo. The restriction enzyme sites used are listed and a SalI and XbaI sites are represented by a (S) and (X) respectively.

UPF1	ATGTCGTATGTTGCACATGTGTTGGTGCTGGTGATAAGCGCTT
UPF1-D1	ATGTCGTATGTTGCACATGTGTTGATGCTGGTGATAAGCGCTT
UPF1-D1 swap sequence	ATGTCGTATGTTGCACATGTGTTGATGCTGGTGATAAGCGCTT

Figure 25. Partial sequences of the wild-type *UPF1* and corresponding sequences in a dominanat-negaitive form of *UPF1* (D1). The *UPF1* and *UPF1-D1* sequence are described in Leeds et al., 1991; *UPF1-D1* swap sequence was deduced by sequencing p73+UPF1-D1/-5'3' with the primer UPF1.bak.

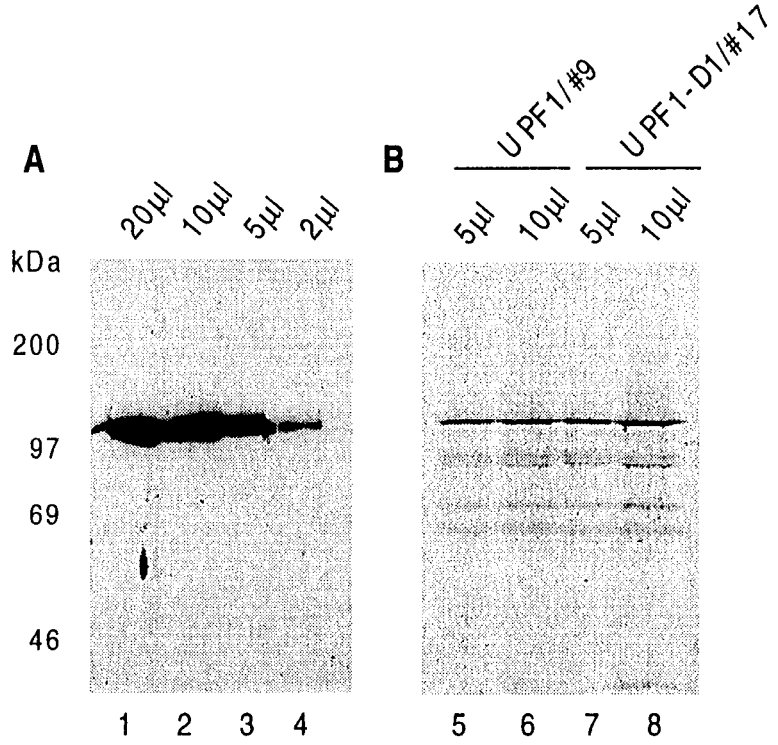


Figure 26. Western blot analysis of yeast cell lysates and UPF1 IVTL products. **(A)** Various amounts of total yeast lysate (as indicated) and **(B)** 5 or 10 µl of whole UPF1 or UPF1-D1 IVTL product were separated on a reducing 10% SDS/PAG. The gel was transferred to nitrocellulose and incubated with anti-UPF1 rabbit antisera (from Jacobson) at 1:200. The rabbit anti-UPF1 antibodies were detected using a HRP-labled goat anti-rabbit IgG (1:7000) followed by ECL detection. The total yeast lysate western was exposed for 5 minutes to film and the UPF1 IVTL western was exposed for 30 minutes.

Expression of UPF1 and UPF1-D1 in Mouse B cells Containing a Nonsense mRNA

To test whether either the wild-type *UPF1* or the dominant-negative *UPF1* interfere with a putative mammalian *UPF1* homologue, I individually transfected the *UPF1* or *UPF1-D1* expression vectors into a mouse B cell hybridoma that contains a nonsense codon within the second exon of the Ig μ heavy chain mRNA (indicated with an X Figure 23) (VXH, Jäck et al., 1989). VXH cells stably transfected with the *UPF1*, *UPF1-D1*, as well as an empty vector were screened by northern blot analysis for their ability to express the *UPF1* or *UPF1-D1* mRNA. Northern blots revealed the same size band in VXH transfected cells and yeast cells (Figure 27, lanes 1-7 and 10-15). From this I concluded that my transfected *UPF1* and *UPF1-D1* genes can be transcribed into mRNA. The same northern blot was also subjected to probing with a mouse μ cDNA probe as well as a GAPDH probe to control for total RNA loading. The levels of hybridization of these probes were quantitated using Betascope blot analysis. After normalization for RNA loading I found that the level of μ nonsense mRNA was unchanged in cells transfected with the *UPF1-D1* expression vector when compared to the negative control (VXH with vector alone) (Table 7). To test whether the *UPF1* and *UPF1-D1* mRNAs can be translated, I performed western blot analysis of the transfected cells. I found that I could also detect a protein of the same molecular weight in my *UPF1-D1* expressing cells (Figure 27, lanes 2, 4, and 14; and Figure 28 1-3) using an anti-UPF1 antibody (gift from A.

Figure 27. Northern blot analysis of VXH cells stably transfected with UPF1-D1 expression vector and vector alone. 10μg of total RNA from VXH (lab nomenclature: GAMO 12.8.10) stably transfected with UPF1-D1 (lanes 2, 3, 4, 5, 6, 7, 11, 12, 13, 14, 15) and vector alone (β-actin) transfectants (lanes 8, and 16) as well as the VXH parent cell line FH (lab nomenclature: LOCB 83.13.13.1) (lanes 9, and 17) were subjected to northern blot analysis as described in materials and methods and first, probed with a mouse μ cDNA, and second, a UPF1 probe. RNA from yeast Y06-15C in lanes 1, and 10 served as a positive control for UPF1 hybridization. To control for RNA loading, the blot was finally hybridized with a rabbit GAPDH cDNA probe (data not shown).

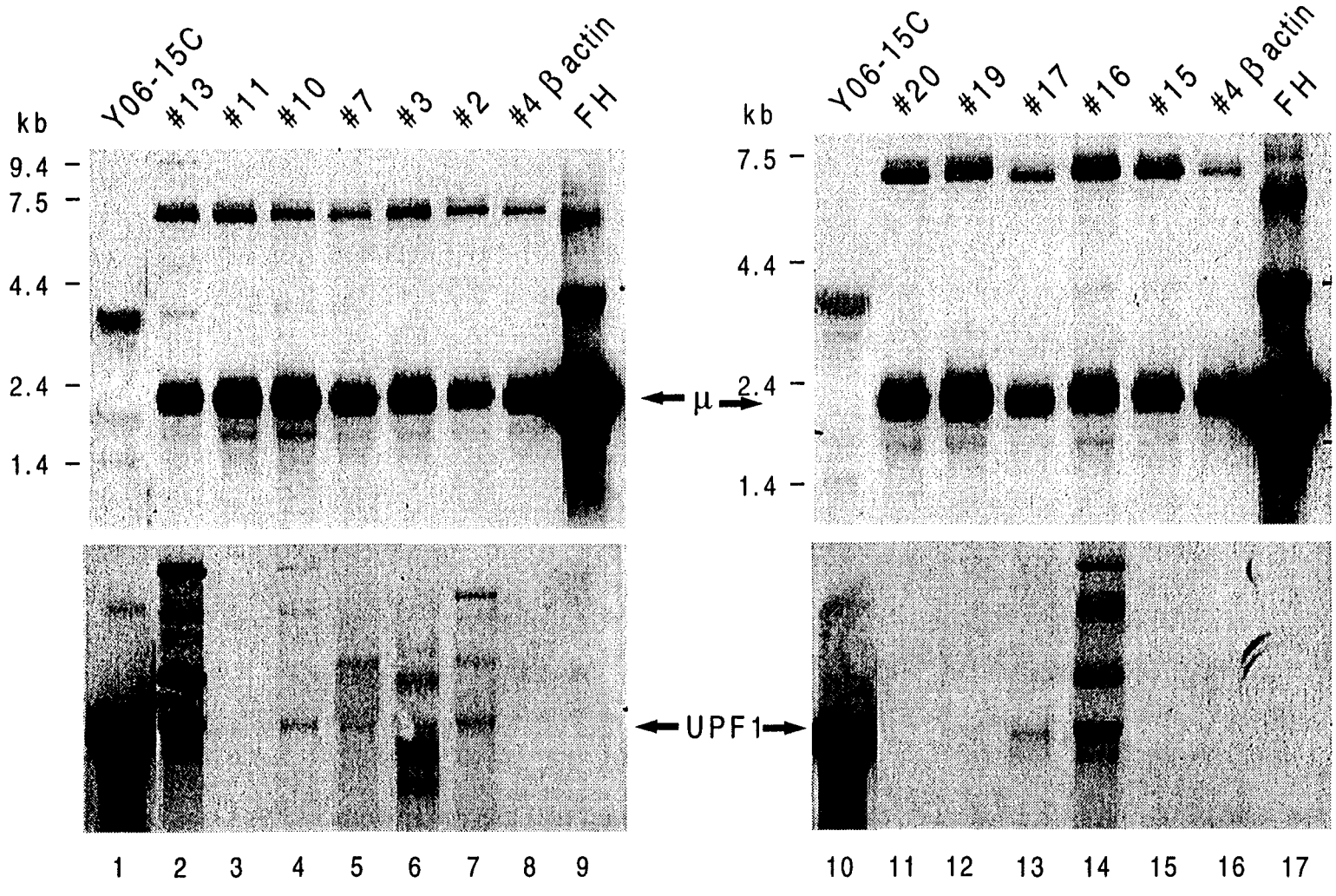

kb
Y06-15C
#13
#11
#10
#7
#3
#2
#4
β actin
FH
9.4
7.5
4.4
2.4
1.4
μ
UPF1
kb
Y06-15C
#20
#19
#17
#16
#15
#4
β actin
FH
7.5
4.4
2.4
1.4
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17

Table 7. --Quantitation of Northern Blot Analysis in Figure 27[a]

Hybridomas	RNA loaded	μ	pre-μ	GAPDH	rel [μ] [μ/GAPDH]	rel [pre-μ] [pre-μ/GADPH]	% μ % VXH	% pre-μ %VXH
		[CPM]	[CPM]	[CPM]				
FH Blot A	5 μg	455.5	6.3	8.2	55.5	0.77	2000%	154%
VXH/β-actin (#4)	5 μg	21.2	3.9	8.0	2.7	0.50	**100%**	**100%**
VXH/UPF1-D1 (#2)	5 μg	12.7	4.3	9.3	1.3	0.46	48%	92%
VXH/UPF1-D1 (#3)	5 μg	21.5	6.7	10.4	2.0	0.64	74%	128%
VXH/UPF1-D1 (#7)	5 μg	16.3	4.1	14.7	1.1	0.28	40%	56%
VXH/UPF1-D1 (#10)	5 μg	24.8	5.1	10.2	2.4	0.50	88%	100%
VXH/UPF1-D1 (#11)	5 μg	23.6	6.5	11.2	2.9	0.58	107%	116%
VXH/UPF1-D1 (#13)	5 μg	13.5	5.5	16.0	0.8	0.34	29%	68%
FH Blot B	5 μg	245.5	5.7	7.8	31.5	0.73	1350%	160%
VXH/β-actin (#4)	5 μg	11.9	2.4	6.4	2.3	0.45	**100%**	**100%**
VXH/UPF1-D1 (#15)	5 μg	10.5	6.6	8.6	1.1	0.77	48%	171%
VXH/UPF1-D1 (#16)	5 μg	13.4	8.7	10.5	0.9	0.82	39%	182%
VHX/UPF1-D1 (#17)	5 μg	9.8	4.0	12.8	0.5	0.31	21%	68%
VXH/UPF1-D1 (#19)	5 μg	22.2	7.7	13.9	0.9	0.55	40%	122%
VXH/UPF1-D1 (#20)	5 μg	20.7	7.8	10.9	1.4	0.71	61%	157%

[a]Radioactivity of respective bands in each Northern blot (CPM) was determined by using a betascope blot analyzer. Relative steady-state level of respective RNA was calculated by dividing CPM of μ by that of GAPDH in the same lane. % [μ] is the percent of [μ] relative to that of untransfected hybridoma FH by setting FH (cells containing the same μ gene as VXH cells except that the stop codon TAG reverted to AAG) value equal to 100%.

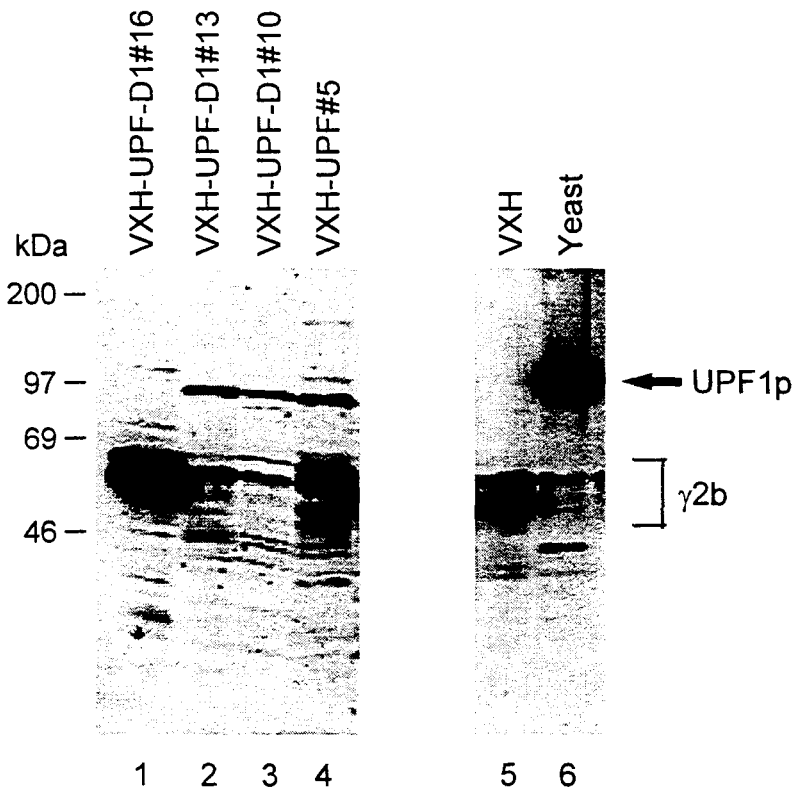


Figure 28. Western blot of protein lysates of VXH cells stably transfected with a *UPF1* or *UPF1-D1* expression vector. Protein from 8 X 10^5 cells was separated by reducing SDS/PAGE, transferred, and probed using an rabbit anti-UPF1 peptide antiserum (1:200). The antibody-antigen complex was detected using a HRP labled-goat anti-IgG antiserum (1:10,000) followed by ECL detection. The bands were detected by fluorography using a 5 minute exposure. Lanes 1-3, *UPF1-D1 transfected cells;* lane 4, *UPF1* transfectants. Lane 5, untransfected VXH and lane 6, total yeast lysate. Bands detected within the bracketed region are mouse γ2b heavy chains that cross react with the goat anti-rabbit IgG secondary antibody.

Jacobson). It is possible that the yeast Upf1p or Upf1-D1p folded at 37°C are unable to act in a dominant-negative fashion. Therefore, I repeated the northern blot analysis with cells that were grown at 30°C, a temperature at which yeast cells are normally cultured in the lab. Northern blot analysis of these transfectants revealed the same results as obtained with cells grown at 37°C (data not shown). From these results, I conclude that the amount of Upf1-D1p, or wild-type Upf1p that is synthesized in stable VXH transfectants does not result in a decrease of nonsense μ mRNA.

Conclusions

If mammalian cells contain a *UPF1*-Like protein, I expected that the expression of yeast Upf1-D1p in a mammalian cell could result in increased levels of nonsense mRNA. If this is the case, it would be the first evidence that a *UPF*-Like system exists in mammalian cells. To test this hypothesis, cells containing a μ nonsense codon-containing mRNA were transfected with a construct designed to promote high level expression of Upf1-D1p, a dominant-negative form of Upf1p. Stable transfectants expressed the *UPF1-D1* mRNA and low levels of Upf1-D1p. No change in nonsense μ mRNA was observed. This result could be due to a number of reasons. First, the level of Upf1-D1p produced was not high enough to interfere with the mammalian nonsense mRNA reduction system. Second, the levels of Upf1-D1p were sufficient to have a presumed effect but a *UPF*-Like system does not exist in mammalian cells.

Search Mammalian DNA Databases With the Yeast UPF1 Amino Acid Sequence and Determine Whether Identified Genes are Structural and Functional Homologues of Yeast UPF1.

To identify human or other mammalian homologues of genes that have been previously cloned in lower organisms, one could compare the cloned sequence to mammalian nucleic acid and protein databases. If a human gene fragment is found, it could then be used to isolate the complete gene from a DNA library. A complete gene could then be sequenced and tested for its function as discussed below.

Using the XREFdb to Search for Mammalian Homologues of the Yeast *UPF1* Gene

Recently a number of new search programs and databases have been developed for researchers who want to find a human, rat, or mouse homologue of genes cloned from lower eukaryotic organisms. One such search program is the XREFdb program (Basset et al., 1995) which is accessible via the world wide web (http://www.ncbi.nlm.nih.gov/XREFdb/). The XREFdb provides BLAST similarity searches (TBLASTX; Altschul et al., 1990) with an amino acid sequence provided by the user. XREFdb searches databases that contain Expressed Sequence Tags (EST) (Boguski et al., 1993) and Sequence Tagged Sites (STS) (Green et al., 1989) of cloned DNA and cDNA fragments. The query sequence submitted to the XREFdb is compared once a month with the rapidly

and ever expanding database of DNA sequences generated from the human and mouse genome projects and a report of the identified genes is sent to the user who submitted the search.

To identify mammalian homologues of the *S. cerevisiae* gene *UPF1*, I submitted the Upf1p sequence (SwissProt accession #P30771) to the XREFdb for the first time in September 1995. In both September and October 1995, XREFdb E-mail reports were sent to me with no significant matches. In November 1995, a XREFdb report was received that contained a number of alignments between amino acid sequences deduced from human EST cDNAs clones and the Upf1p.

Analysis of Clones Whose Sequence Matched That of UPF1

The original data report that was sent in November, 1995, by the XREFdb is shown in Figure 29. When I located the regions that matched with the EST sequences in the *UPF1* sequence, I found that all EST sequences aligned with the RNA helicase region of the Upf1p (Figure 30). Further analysis of the alignments revealed three subsets of matched sequences. The first subset contains the first two genes identified in the XREFdb report (GenBank accession #F06433 and #R13609). These two human EST cDNAs, which were derived independently from same library (a human fetal brain cDNA library constructed by Benito Soares, M. Fatima Bonaldo, Hillier, L., The WashU-Merck EST Project, unpublished, 1995) share a large amount of identical amino acid residues (Figure 29). This suggests that they originated from the same transcript.

Figure 29. XREFdb announcement that my UPF1 query sequence has been matched to a number of recently sequenced human cDNA EST clones. Sbjct indicates the marked EST sequence; query, the submitted UPF1 sequence; positives, the number of identical and similar amino acid matches between the indicated sbjct and query.

```
EST Similarity Search Report:  (TBLASTN vs. human/mouse/rat dbEST subset)
-----------------------------------------------------------------------------
Gene name(s): NAM7/UPF1  Searched: 28 Oct 95
-----------------------------------------------------------------------------
                GenBank                                              Mapped?
New?  NCBI_ID:  Accession: P value:   N:  Matrix:   Hiscore:  (Probe #)
-----------------------------------------------------------------------------

New   122095    F06433     4.1e-38    2   BLOSUM62    183     Yes(#165)*

Organism:       H.sapiens
Library:        normalized infant brain cDNA
Source:         Genethon
E-mail:         genexpress@genethon.fr
Phone:          33169472800          FAX: 33160778698

Score = 183 (83.9 bits), Expect = 4.1e-38, Sum P(2) = 4.1e-38
 Identities = 37/68 (54%), Positives = 43/68 (63%), Frame = +2

Query:   624 LISLGHVPIRLEVQYRMNPYLSEFPSNMFYEGSLQNGVTIEQRTVPNSKFPWPIRGIPMM 683
             L+ LG  P RL+VQYRM+  LS FPSN+FYEGSLQNGVT   R      F WP    PM
Sbjct:   146 LVXLGIRPXRLQVQYRMHSALSAFPSNIFYEGSLQNGVTAADRVKKGFDFQWPQPDKPMF 325

Query:   684 FWANYGRE 691
             F+   G+E
Sbjct:   326 FYVTQGQE 349

 Score = 174 (79.8 bits), Expect = 4.1e-38, Sum P(2) = 4.1e-38
 Identities = 32/47 (68%), Positives = 40/47 (85%), Frame = +3

Query:   576 QASEPECLIPIVKGAKQVILVGDHQQLGPVILERKAADAGLKQSLFE 622
             Q +EPEC++P+V G KQ+ILVGDH QLGPV++ +KAA AGL QSLFE
Sbjct:     3 QXTEPECMVPVVLGGKQLILVGDHCQLGPVVMCKKAAKAGLSQSLFE 143


New   184188    R13609     4.6e-30    2   BLOSUM62    165       No

Organism:       H.sapiens
Library:        Soares infant brain 1NIB
Source:         Wilson RK
E-mail:         est@watson.wustl.edu
Phone:          314 286 1800          FAX: 314 286 1810

 Score = 165 (75.7 bits), Expect = 4.7e-30, Sum P(2) = 4.7e-30
 Identities = 33/52 (63%), Positives = 37/52 (71%), Frame = +2

Query:   624 LISLGHVPIRLEVQYRMNPYLSEFPSNMFYEGSLQNGVTIEQRTVPNSKFPW 675
             L+ LG  PIRL+VQYRM+P LS FPSN+FYEGSLQNGVT   R      F W
Sbjct:   143 LVVLGIRPIRLQVQYRMHPALSAFPSNIFYEGSLQNGVTAADRVKKGFDFQW 298

 Score = 133 (61.0 bits), Expect = 4.6e-30, Sum P(2) = 4.6e-30
 Identities = 24/37 (64%), Positives = 32/37 (86%), Frame = +1

Query:   578 SEPECLIPIVKGAKQVILVGDHQQLGPVILERKAADA 614
             +EP C++P+V GAKQ+ILVGDH QLGPV++ +KAA A
Sbjct:     7 TEPXCMVPVVLGAKQLILVGDHCQLGPVVMCKKAAKA 117


New   271744    H11830     2.2e-11    3   BLOSUM62    83        No

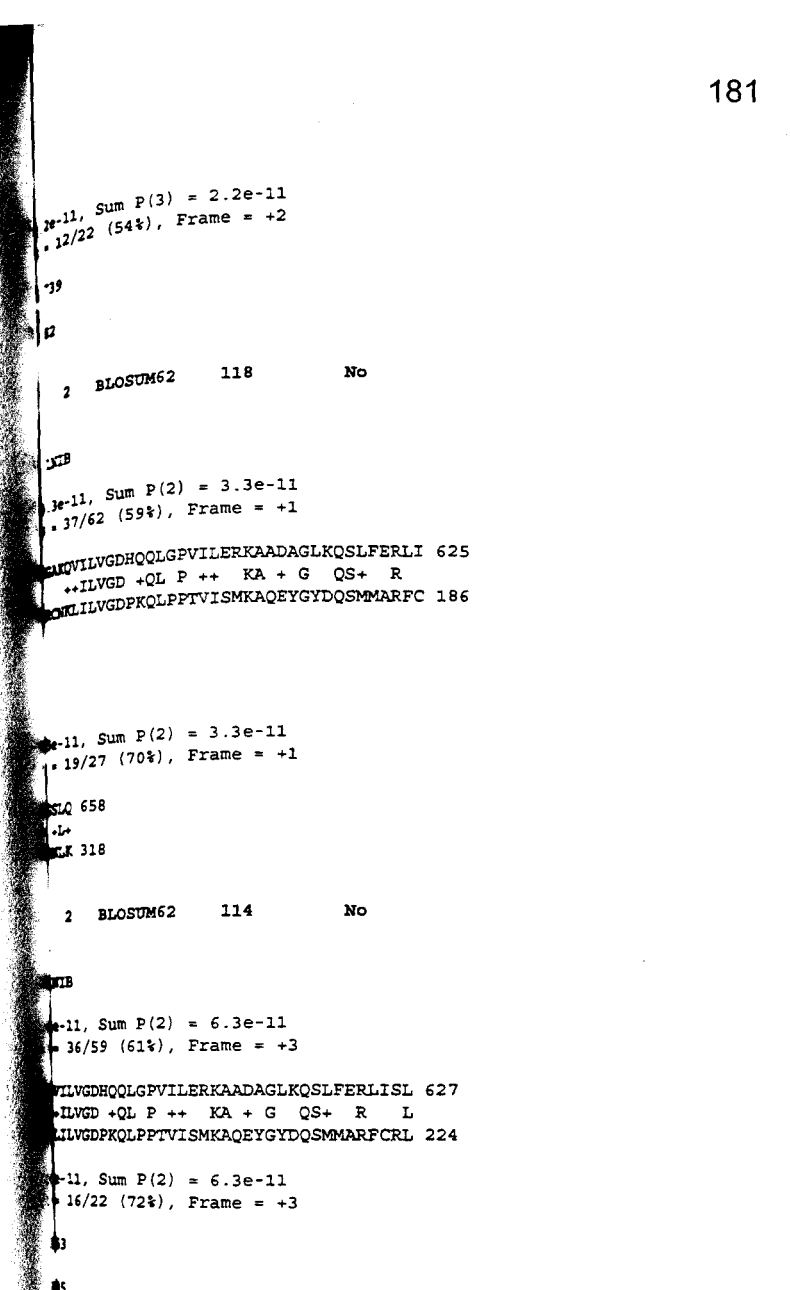
Organism:       H.sapiens
Library:        Soares infant brain 1NIB

 Score = 83 (38.1 bits), Expect = 2.2e-11, Sum P(3) = 2.2e-11
 Identities = 17/27 (62%), Positives = 22/27 (81%), Frame = +2

Query:   758 EVASVDAFQGREKDYIILSCVRANEQQ 784
             EV +VDAFQGR+KD +I++ VRAN  Q
Sbjct:   167 EVDTVDAFQGRQKDCVIVTXVRANSIQ 247

 Score = 71 (32.6 bits), Expect = 2.2e-11, Sum P(3) = 2.2e-11
 Identities = 15/23 (65%), Positives = 19/23 (82%), Frame = +3

Query:   793 RRLNVGLTRAKYGLVILGNPRSL 815
             +RLNV +TRAKY L ILG+ R+L
Sbjct:   276 QRLNVTITRAKYSLFILGHLRAL 344
```



EST clones and their names used in the lab.

Insert Size in kb (approximate)	Lab Designation
1.5	Clone 1 / SAM1
1.3	Clone 2 / SAM2
1.6	Clone 3 / SAM3
N/A	Clone 4 / SAM4

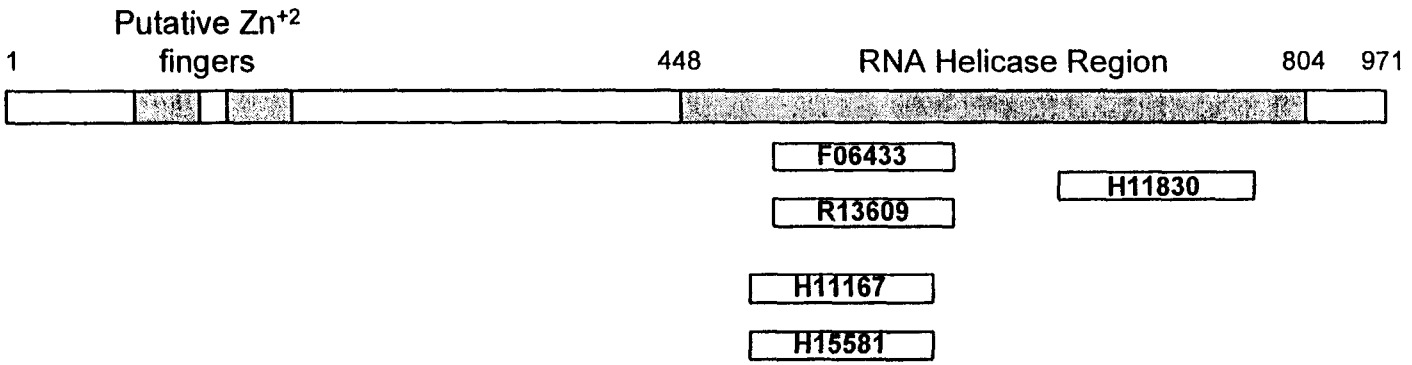


Figure 30. Schematic representation of the yeast UPF1 protein and alignments with the putative translation products deduced from from cDNA EST clones identifed by the XREFdb and shown in Figure 29. Amino acid residues in UPF1 that border the RNA Helicase region are indicated.

I, therefore, decided to obtain the clone that was the most accessible (GenBank accession #R13609). The second subset contained only clone H11830 (GenBank accession #H11830). The third and final subset contained clones H15581 and H11176 (GenBank accession #H15581, #H11167). These two human EST cDNAs overlap completely. Therefore, only clone H11167 was used in all subsequent experiments. The human EST cDNA clones mentioned above were requested and upon arrival, all four clones were renamed (Table 8). The insert size of clones 1, 2, and 3 was determined by restriction enzyme digestion. Even though the GenBank sequence of each clone was between 250 and 400 nt long, the actual insert size of the clones was between 1.3-kb and 1.6-kb in length. Clone 1 (R13609) had an insert size of approximately 1.5 kb. To verify the identity of clone 1, both ends of this clone were sequenced. The 5' sequence of clone 1 could be aligned with the published GenBank sequence of clone 1 (#R13609) (Figure 31). The 3' end of the clone contained nucleotides compatible to the oligomer used to generate the first strand cDNA synthesis during the cDNA library construction (data not shown). Therefore, my clone 1 contains a 1.5-kb insert whose 5' sequence is identical to that published in GenBank file #R13609. Next, I isolated the 1.5-kb insert from the plasmid and used it as a probe in northern blot analysis of total human RNA. Inserts were also prepared from clones 2 and 3 (GenBank clones #H11830 and #H11167, respectively) and used as probes for northern blot analysis. However, the sequences of inserts 2 and 3 were not confirmed by DNA sequencing. The

R13609 = Sequence obtained from GenBank file #R13609

SEA = Sequence by SEA

```
R13609  CCAGGCACCGAGCCGNAGTGCATGGTTCCCGTGGTCCTCGGGGCCAAGC
                                                      ||||||
SEA                                                   CCAAGC

R13609 AGCTGATCCTTG-AGGCGACCACTGCCAGCTGGGCCCAGTGGTGATGTGC
       ||||||||||||  |||||||||||||||||  ||||||||||||||||  ||
SEA    AGCTGATCCTTGTAGGCGACCACTGCCAGCAGGGCCCAGTGGTGATG-GC

R13609 AAGAAGGCGGCCAAGGCGGNCTGTCACAGT-CGCTCTTCGAGNCCTGGTG
       ||||||||||||||| ||| ||||||||||  |   |
SEA    AAGAAGGCGGCCAAG-CGG-CTGTCACAGTCCG-T

R13609 TGCTGGGCATCCGGCCCATCCGCCTGCAGGTCCAGTACCGGATGCACCC

R13609 AATGGTGTCACTGCAGCGGATCGTGTGAAGAAGGGATTTGATTTCCAGT

R13609 CAAGAGGGAGATTTGCCAGTTTGGGGCAACTTCTTACCTTGAACAGGGA

R13609 CC
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Figure 31. Alignment of nucleotide sequences from the 5' end of clone 1 (SEA) and GenBank clone #R13609. Sequence of the 5' end of clone R13609 was obtained by double stranded sequencing of plasmid containing insert of clone 1 by the Sanger didioxy method (Sanger et al., 1977) using primer M13.back primer. Alignment errors may be due to errors in the automated sequencing of the EST clone or by incorrect reading of data by (SEA).

results from these three northern are shown in Figure 32. Northern blot analysis using clone 1 revealed in human cells, two bands of 3.6 and 5.5 kb, whereas probing blots with both clones 2 and 3 revealed in human cells bands of 8.0 and 11.0 kb in size. Interestingly, in mouse cells, clone 1 detected a weak band of 4.5 kb in size and clone 2 detected a weak band of 10.8 kb. Whether or not these bands represent murine homologues of human clone 1 and 2 remains to be seen. From these experiments, I conclude that clone 1 might represent a homologue of the yeast *UPF1* gene because the size of the transcripts (3.7- and 5.5-kb) are closest in size to those encoded by *UPF1* (3.2-kb).

Isolation of a Human cDNA Clone Using a Clone 1 Probe

To isolate a full length human cDNA representing the human clone 1 cDNA fragment (R13609), I screened approximately 2.5 X 10^5 individual plaques of a human oligo-d(T)-primed cDNA library prepared from HeLa cell RNA (Elledge et al., 1991) with the isolated 1.5-kb fragment of clone 1 under high stringency conditions. Seven hybridizing phage clones were identified, plaque purified, and used to infect an *E. coli* strain able to excise a yeast/*E. coli* shuttle vector from the phage DNA. The shuttle vector can be used to express the cDNA from an inducible promoter in yeast cells (Figure 33). Plasmids could be excised from seven (clone numbers 8.1.1/2, 11.1.1/2, 11.2.1/2, 13.1.1/2, 15.1.1/2, 21.1.1/2, 22.1.1/2) of seven clones and the size of their cDNA inserts was determined by restriction digestion (Appendix Figure 1.A). Six of the seven

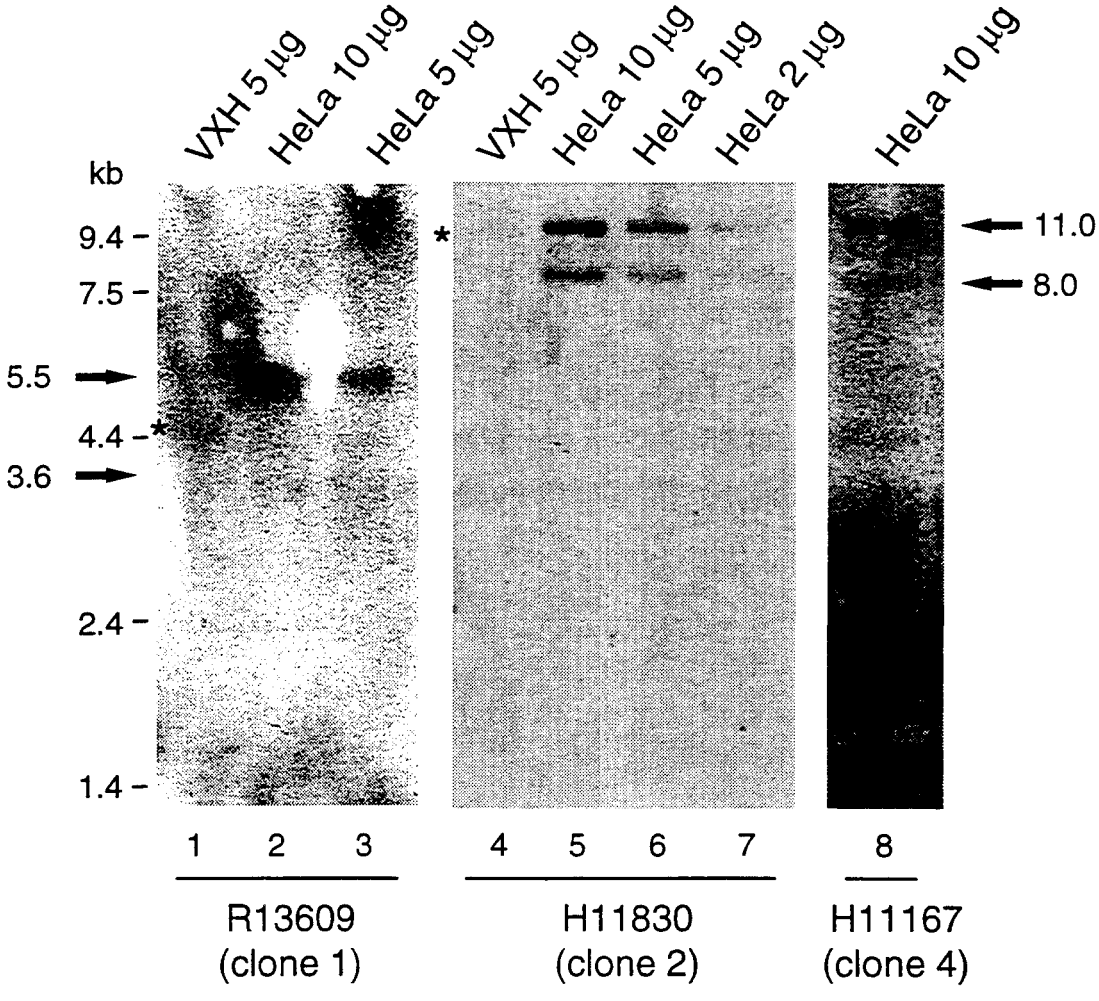

Figure 32. Northern blot analysis of human and mouse RNA using probes isolated from human cDNA EST clones. Indicated amounts of total RNA were subjected to northern blot analysis using the probes called clone 1 (R13609), 2 (H11830) and 3 (H11167). Arrows indicate the position and size of RNA bands detected in human cells. An asterisk indicates faint bands detected in mouse cells that are 11.0-kb band with the H11830 probe (lane 4) and a 4.6-kb band with the R13609 probe (lane 1).

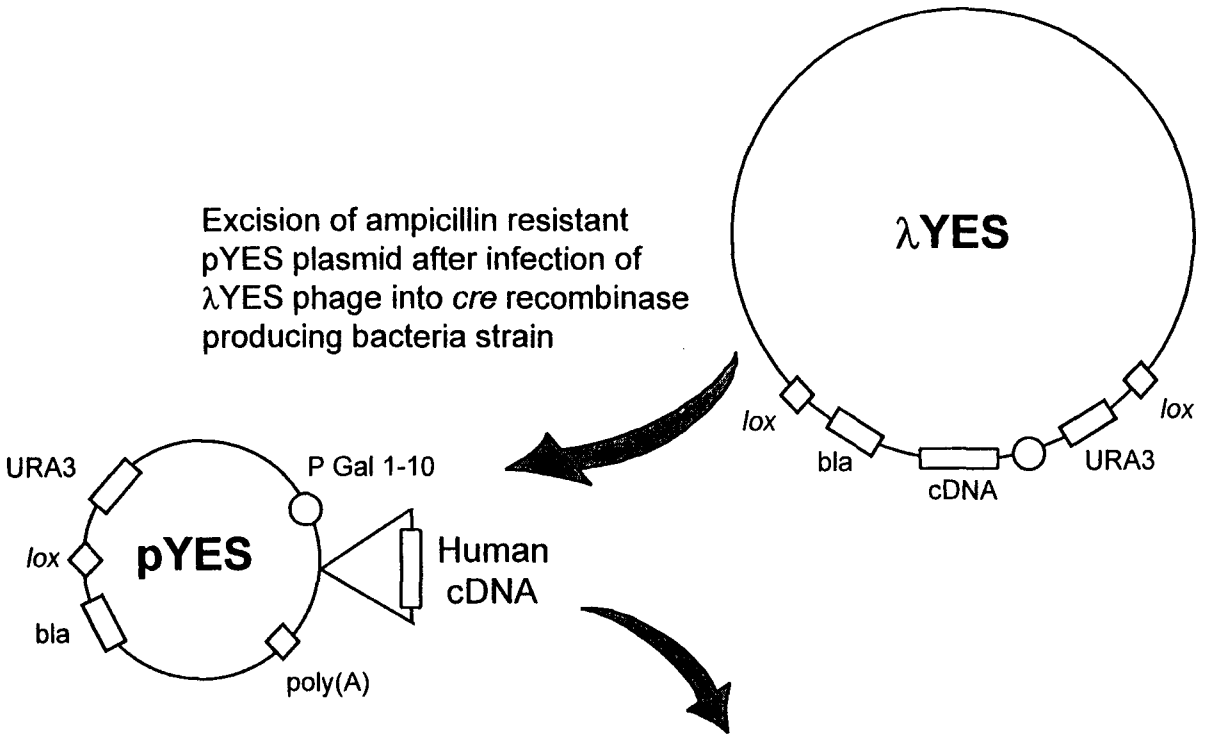

Figure 33. Schematic diagram of pYES plasmid excision from phage λYES and subsquent test of human cDNA for complementing ability.

clones contained detectable inserts. One clone (clone 11.2.1/2) contained an insert of 3.6-kb (called clone 3.6)., whereas the other clones contained smaller inserts (Appendix Figure 1.A, 1.C). Restriction mapping revealed that three of the clones with smaller inserts (21.1.1, 8.1.1, 13.1.1) overlapped with the insert of clone 3.6 (Appendix Figure 1.B, 1.D). These clones were not examined further.

When I sequenced the 3' end of clone 3.6, I found that the nucleotide sequence was identical to that of the original clone 1 (GenBank clone #R13609) (data not shown). When I used the 3.6-kb insert as a probe in a northern blot analysis of human total HeLa RNA, I detected bands of 5.5- and 3.6-kb (Figure 34). Because the original 1.5-kb clone 1 probe also detected 3.6-kb and 5.5-kb bands in HeLa RNA (Figure 32), I conclude that I have cloned a 3.6-kb cDNA that very likely represents a full length cDNA of the original 1.5-kb clone 1 probe.

Sequence Analysis

The insert of the 3.6-kb clone (clone 3.6) was sequenced by Sanger dideoxy chain termination method (Sanger et al., 1977) and primer-walking (Slightom et al., 1994) with the ABI Cycle Sequencing kit from Perkin Elmer (Branchburg, NJ) and an ABI automated DNA sequencer. The sequence of clone 3.6 was 3602 bp in length and contains the poly(A)-signal sequence AATAAA and from nucleotide 176 to nucleotide 3529 a 3354-bp open reading frame (ORF) that could encode a 123-kDa polypeptide (Figure 35). The first amino (N)-terminal ATG codon of clone 3.6 is very likely the true translational

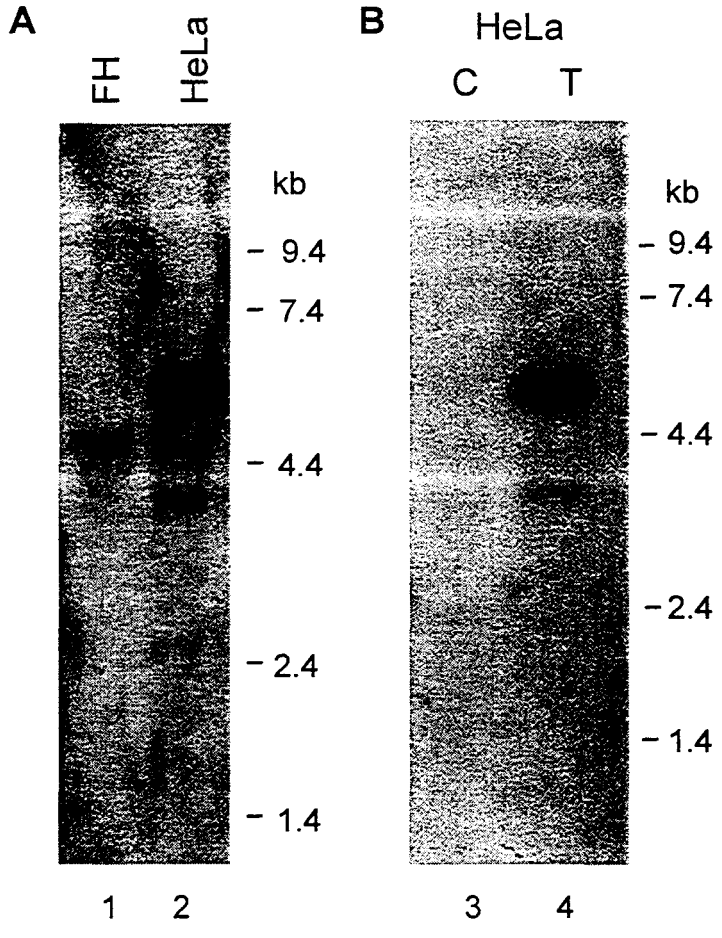

Figure 34. Northern blot analysis of total and cytoplasmic RNA using the 3.6 clone probe. **(A)** Northern blot of 5 μg of total RNA from the mouse B cell hybridoma FH (Lane 1) and human endothelial cell line HeLa (lane 2). Blot was exposed to film 2 days at -70°C with one enhancing screen. **(B)** Northern blot of 10 μg of cytoplasmic (C, lane 3) and total (T, lane 4) HeLa RNA. Blot was exposed to film 3 days at -70°C with one enhancing screen.

Figure 35. Nucleotide and predicted amino acid sequence of the human NORF1 gene.

```
gcggcggctcggcactgttacctctcggtccggctggcgccgcgggcggtttggtcctttccgggcgcgcgggggcgaca 80

gcggcagcgacccgaggcctgcggcctaggcctcagcgcggcggcgggctcgagtgcagcgcggaaccggcccgagggcc 160

ctacccggaggcaccatgagcgtggaggcgtacgggcccagctcgcagactctcactttcctggacacggaggaggccga 240
                  M  S  V  E  A  Y  G  P  S  S  Q  T  L  T  F  L  D  T  E  E  A  E 22

gctgcttggcgccgacacacagggctccgagttcgagttcaccgactttactcttcctagccagacgcagacgccccccg 320
  L  L  G  A  D  T  Q  G  S  E  F  E  F  T  D  F  T  L  P  S  Q  T  Q  T  P  P  48

gcggccccggcggcccgggcggtggcggcgcgggaagcccgggcggcgcgggcgccggcgctgcggcgggacagctcgac 400
G  G  P  G  G  P  G  G  G  G  A  G  S  P  G  G  A  G  A  G  A  A  A  G  Q  L  D  75

gcgcaggttgggcccgaaggcatcctgcagaacggggctgtggacgacagtgtagccaagaccagccagttgttggctga 480
 A  Q  V  G  P  E  G  I  L  Q  N  G  A  V  D  D  S  V  A  K  T  S  Q  L  L  A  E 102

gttgaacttcgaggaagatgaagaagacacctattacacgaaggacctccccatacacgcctgcagttactgtggaatac 560
  L  N  F  E  E  D  E  E  D  T  Y  Y  T  K  D  L  P  I  H  A  C  S  Y  C  G  I  128

acgatcctgcctgcgtggtttactgtaataccagcaagaagtggttctgcaacggacgtggaaatacttctggcagccac 640
H  D  P  A  C  V  V  Y  C  N  T  S  K  K  W  F  C  N  G  R  G  N  T  S  G  S  H  155

attgtaaatcaccttgtgagggcaaaatgcaaagaggtgaccctgcacaaggacgggcccctgggggagacagtcctgga 720
 I  V  N  H  L  V  R  A  K  C  K  E  V  T  L  H  K  D  G  P  L  G  E  T  V  L  E 182

gtgctacaactgcggctgtcgcaacgtcttcctcctcggcttcatcccggccaaagctgactcagtggtggtgctgctgt 800
  C  Y  N  C  G  C  R  N  V  F  L  L  G  F  I  P  A  K  A  D  S  V  V  V  L  L  208

gcaggcagccctgtgccagccagagcagcctcaaggacatcaactgggacagctcgcagtggcagccgctgatccaggac 880
C  R  Q  P  C  A  S  Q  S  S  L  K  D  I  N  W  D  S  S  Q  W  Q  P  L  I  Q  D  235

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 R  C  F  L  S  W  L  V  K  I  P  S  E  Q  E  Q  L  R  A  R  Q  I  T  A  Q  Q  I 262

caacaagctggaggagctgtggaaggaaaaccccttctgccacgctggaggacctggagaagccgggggtggacgaggagc 1040
  N  K  L  E  E  L  W  K  E  N  P  S  A  T  L  E  D  L  E  K  P  G  V  D  E  E  288

cgcagcatgtcctcctgcggtacgaggacgcctaccagtaccagaacatattcgggcccctggtcaagctggaggccgac 1120
P  Q  H  V  L  L  R  Y  E  D  A  Y  Q  Y  Q  N  I  F  G  P  L  V  K  L  E  A  D  315

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 Y  D  K  K  L  K  E  S  Q  T  Q  D  N  I  T  V  R  W  D  L  G  L  N  K  K  R  I 342

cgcctacttcactttgcccaagactgactctgacatgcggctcatgcaggggatgagatatgcctgcggtacaaagggg 1280
  A  Y  F  T  L  P  K  T  D  S  D  M  R  L  M  Q  G  D  E  I  C  L  R  Y  K  G  368

accttgcgcccctgtggaaagggatcggccacgtcatcaaggtccctgataattatggcgatgagatcgccattgagctg 1360
D  L  A  P  L  W  K  G  I  G  H  V  I  K  V  P  D  N  Y  G  D  E  I  A  I  E  L  395

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 R  S  S  V  G  A  P  V  E  V  T  H  N  F  Q  V  D  F  V  W  K  S  T  S  F  D  R 422

gatgcagagcgcattgaaaacgtttgccgtggatgagacctcggtgtctggctacatctaccacaagctgttgggccacg 1520
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E  V  E  D  V  I  T  K  C  Q  L  P  K  R  F  T  A  Q  G  L  P  D  L  N  H  S  Q  475

gtttatgccgtgaagactgtgctgcaaagaccactgagcctgatccagggcccgccaggcacggggaagacggtgacgtc 1680
 V  Y  A  V  K  T  V  L  Q  R  P  L  S  L  I  Q  G  P  P  G  T  G  K  T  V  T  S 502

ggccaccatcgtctaccacctggcccggcaaggcaacgggccggtgctggtgtgtgctccgagcaacatcgccgtggacc 1760
  A  T  I  V  Y  H  L  A  R  Q  G  N  G  P  V  L  V  C  A  P  S  N  I  A  V  D  528

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Q  L  T  E  K  I  H  Q  T  G  L  K  V  V  R  L  C  A  K  S  R  E  A  I  D  S  P  555

gtgtcttttctggccctgcacaaccagatcaggaacatggacagcatgcctgagctgcagaagctgcagcagctgaaaga 1920
 V  S  F  L  A  L  H  N  Q  I  R  N  M  D  S  M  P  E  L  Q  K  L  Q  Q  L  K  D 582

cgagactggggagctgtcgtctgccgacgagaagcggtaccgggccttgaagcgcaccgcagagagagagctgctgatga 2000
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acgcagatgtcatctgctgcacatgtgtgggcgccggtgacccgaggctggccaagatgcagttccgctccattttaatc 2080
N  A  D  V  I  C  C  T  C  V  G  A  G  D  P  R  L  A  K  M  Q  F  R  S  I  L  I  635
```

```
gacgaaagcacccaggccaccgagccggagtgcatggttcccgtggtcctcggggccaagcagctgatccttgtaggcga 2160
 D  E  S  T  Q  A  T  E  P  E  C  M  V  P  V  V  L  G  A  K  Q  L  I  L  V  G  D 662

ccactgccagctgggcccagtggtgatgtgcaagaaggcggccaaggccgggctgtcacagtcgctcttcgagcgcctgg 2240
  H  C  Q  L  G  P  V  V  M  C  K  K  A  A  K  A  G  L  S  Q  S  L  F  E  R  L  688

tggtgctgggcatccggcccatccgcctgcaggtccagtaccggatgcaccctgcactcagcgccttcccatccaacatc 2320
V  V  L  G  I  R  P  I  R  L  Q  V  Q  Y  R  M  H  P  A  L  S  A  F  P  S  N  I  715

ttctacgagggctccctccagaatggtgtcactgcagcggatcgtgtgaagaagggatttgacttccagtggccccaacc 2400
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  D  K  P  M  F  F  Y  V  T  Q  G  Q  E  E  I  A  S  S  G  T  S  Y  L  N  R  T  768

aggctgcgaacgtggagaagatcaccacgaagttgctgaaggcaggcgccaagccggaccagattggcatcatcacgccc 2560
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gatcgccagtgtggacgcctttcagggacgcgagaaggacttcatcatcctgtcctgtgtgcgggccaacgagcaccaag 2720
  I  A  S  V  D  A  F  Q  G  R  E  K  D  F  I  I  L  S  C  V  R  A  N  E  H  Q  848

gcattggctttttaaatgaccccaggcgtctgaacgtggccctgaccagagcaaggtatggcgtcatcattgtgggcaac 2800
G  I  G  F  L  N  D  P  R  R  L  N  V  A  L  T  R  A  R  Y  G  V  I  I  V  G  N  875

ccgaaggcactatcaaagcagccgctctggaaccacctgctgaactactataaggagcagaaggtgctggtggaggggcc 2880
 P  K  A  L  S  K  Q  P  L  W  N  H  L  L  N  Y  Y  K  E  Q  K  V  L  V  E  G  P 902

gctcaacaacctgcgtgagagcctcatgcagttcagcaagccacggaagctggtcaacactatcaacccgggagcccgct 2960
  L  N  N  L  R  E  S  L  M  Q  F  S  K  P  R  K  L  V  N  T  I  N  P  G  A  R  928

tcatgaccacagccatgtatgatgcccgggaggccatcatcccaggctccgtctatgatcggagcagccagggccggcct 3040
F  M  T  T  A  M  Y  D  A  R  E  A  I  I  P  G  S  V  Y  D  R  S  S  Q  G  R  P  955

tccagcatgtacttccagacccatgaccagattggcatgatcagtgccggccctagccacgtggctgccatgaacattcc 3120
 S  S  M  Y  F  Q  T  H  D  Q  I  G  M  I  S  A  G  P  S  H  V  A  A  M  N  I  P 982

catccccttcaacctggtcatgccacccatgccaccgcctggctattttggacaagccaacgggcctgctgcagggcgag 3200
  I  P  F  N  L  V  M  P  P  M  P  P  P  G  Y  F  G  Q  A  N  G  P  A  A  G  R  1008

gcaccccgaaaggcaagactggtcgtgggggacgccagaagaaccgctttgggcttcctggacccagccagactaacctc 3280
G  T  P  K  G  K  T  G  R  G  G  R  Q  K  N  R  F  G  L  P  G  P  S  Q  T  N  L  1035

cccaacagccaagccagccaggatgtggcgtcacagcccttctctcagggcgccctgacgcagggctacatctccatgag 3360
 P  N  S  Q  A  S  Q  D  V  A  S  Q  P  F  S  Q  G  A  L  T  Q  G  Y  I  S  M  S 1062

ccagccttcccagatgagccagcccggcctctcccagccggagctgtcccaggacagttaccttggtgacgagtttaaat 3440
  Q  P  S  Q  M  S  Q  P  G  L  S  Q  P  E  L  S  Q  D  S  Y  L  G  D  E  F  K  1088

cacaaatcgacgtggcgctctcacaggactccacgtaccagggagagcgggcttaccagcatggcggggtgacggggctg 3520
S  Q  I  D  V  A  L  S  Q  D  S  T  Y  Q  G  E  R  A  Y  Q  H  G  G  V  T  G  L  1115

tcccagtattaaaaggtggcggcggaagagctaagcaacgtggcttagtccatcagcatcttattctgggtaataaaaaaa 3600
 S  Q  Y  *                                                                      1118

tg                                                                               3602
```

initiation codon, because it is flanked by sequences that predict strong translational initiation (Kozak, 1994) and is preceded by an in-frame TAG stop codon from nucleotide positions 107 to 109. As mentioned above, clone 3.6 could encode a protein of approximately 123-kDa. To determine if this is the case, *in vitro* transcription-translation (IVTL) was performed using a linearized *NORF1* cDNA plasmid preceded by a T7 polymerase promoter. When radiolabeled IVTL products were subjected to SDS/PAGE, I detected a band with an apparent molecular mass of 130 kDa (Figure 36). This result indicates that the ORF of the 3.6-kb *NORF1* clone (clone 3.6) can be translated into a protein product of approximately 130-kDa. This corresponds well with the calculated molecular mass of 123 kDa. The difference between predicted and apparent molecular weight might be due to the fact that acidic polypeptides run aberrantly in SDS polyacrylamide gels (Koshland, 1985; Bornemann et al., 1995).

Comparison of NORF1 Polypeptide Sequence to Sequences in Databases

When the translated non-redundant GenBank, PDB, SwissProt, and PIR databases (release date: July 22, 1996) was searched with the deduced amino acid sequence of clone 3.6 using the BLAST program (Altschul et al., 1990), the results revealed that the human protein showed the highest degree of homology to yeast *UPF1* (Leeds et al., 1991), which is also known as *NAM7* (Altamura et al., 1992). First, 71% of the human coding sequence from codon 121 to codon

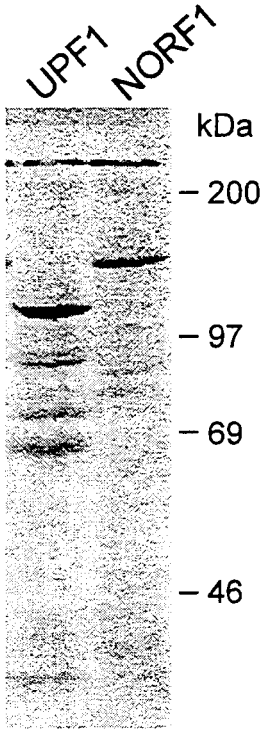


Figure 36. *In vitro* transcription-translation (IVTL) of *UPF1* and *NORF1* RNA transcribed from plasmid DNA. 5 μg of linearized plasmid was subjected to *in vitro* transcription-translation in the presence of ^{35}S-labeled methionine and cystine using the TnT coupled transcription-translation kit (Promega, WI) as suggested by the manufacturer. 10 μl of reaction product was separated by 10% SDS/PAGE and subjected to flurography for 16 hours.

918 is 60% identical and 74% similar to the corresponding *UPF1 (NAM7)* coding sequence from codons 60 to 856. Second, both proteins contain at their amino (N)-terminal ends an acidic stretch followed by a cysteine-rich region and in their middle parts, an RNA helicase region (Figure 37.B).

The RNA helicase region of the human polypeptide from codons 489 to 875 is 63% identical and 85% similar to that of Upf1p (codons 427 to 811) and contains in the same order all seven RNA helicase motifs that are found in Upf1p (Altamura et al., 1992; Leeds et al., 1992), and other group I RNA helicases (Koonin, 1992). Remarkably, the distances between six of the seven RNA helicase consensus motifs are preserved between the human protein and yeast Upf1p and the distances between domains Ib and II differ only by two amino acid residues (Figure 37.B). The BLAST search revealed also that all seven RNA helicase consensus motifs in the human protein are very similar to that of other putative eukaryotic group I RNA helicases (Koonin, 1992) such as *S. cerevisiae SEN1*, mouse MOV-10, the putative *S. pombe UPF1* homologue, the human and mouse μ switch region binding proteins SMBP-2, a putative human DNA helicase, and an insulin enhancer binding protein (Figure 37, Table 9). Thus, I conclude that the human 3.6-kb cDNA clone encodes a putative group I RNA helicase.

In contrast to the seven RNA helicase motifs, which are present in all group I RNA helicases, a cysteine-rich region is found only in the human protein and Upf1p but not in other members of the group I RNA helicase family. The

Figure 37. Sequence comparison of human NORF1 and yeast UPF1. (**A**) Partial complementary nucleotide and entire deduced amino acid sequences of human *NORF1* and comparison with the *S. cerevisiae* UPF1 amino acid sequence (SwissProt accession # P30771). The numbers at the right indicate nucleotide and amino acid positions. Untranslated regions are shown in lowercase and translated regions in uppercase letters. Arrows indicate the region of the *NORF1* nucleotide sequence that was omitted from the figure and can be retrieved from GenBank file #U59323. The start and stop codons of *NORF1* are in bold and the in-frame stop codon upstream of the ATG as well as the poly(A)-addition signal site are underlined and italicized. The glycine/proline-rich regions and the serine/glutamine (SQ) and serine/glutamine/proline (SQP) repeats are underlined. The deduced amino acid sequences of NORF1 and UPF1 (in single letter IUPC code) are shown below the partial nucleotide sequence. In the alignments (top, NORF1 and bottom, UPF1), a period indicates an identical amino acid residue and a forward slash a gap of one amino acid introduced for optimal alignments. Charged amino acids at the N-terminal ends are indicated by + and -. The cysteine-rich putative zinc finger motifs are underlined in bold, a putative tyrosine phosphorylation site is boxed and the seven group I RNA helicase motifs (I, Ib, II-VI) are indicated by gray-shaded boxes. (**B**) Domain maps of human NORF1 and yeast UPF1. The domains are indicated by boxes. Numbers between and below the domains and at the ends indicate amino acid distances, and numbers in the boxes the net charge of the domain. "PG", proline/glycine-rich; "SQP" serine/glutamine and serine/glutamine/proline repeats.

A

```
                                                                    gcggcggctcggc  13
actgttacctctcggtccggctggcgccgcgggcggtttggtcctttccgggcgcgcgggggcgacagcggcagcgacccg  94
aggcctgcggcctaggcctcagcgcggcggcgggctcgagtgcagcgcggaaccggcccgagggccctacccggaggcacc 175
ATGAGC-->  -                       - -- -   -   - - -
 M  S  V  E  A  Y  G  P  S  SQTLTFLDTEEAELLGADTQGSEFEFTDFTLPSQTQTPPGGPGGPGGGGAGSP  62
            -     -        --  +     -  ------          +       +-
GGAGAGAAAGQLDAQVGPEGILQNGAVDDSVAKTSQLLAELNFEEDEEDTYYTKDLPI//HACSYCGIHDPACVVYCNTS 140
 MV.S.SHTPYDISNSPSDVNV.PATQLN.TLVEDDDVDNQL...AQVTETGFRSPSASDNS.A....DSAK..IK..SC  79
       +   -      -       ---- -   --   -   +   -
KKWFCNGRGNTSGSHIVNHLVRAKCKEVTLHKDGPLGETVLECYNCGCRNVFLLGFIPAKADSVVVLLCRQPCASQSSLK 220
......TKNG..S........LSHHNV.S..P.SD..D.........RK.......VS..SEA.......I.../.T//. 156

DINWDSSQWQPLIQDRCFLSWLVKIPSEQEQLRARQITAQQINKLEELWKENPSATLEDLEKPGVDEEPQHVLLRYEDAY 300
NA...TD......E..QL...VAEQ.T.E.K.K..L..PS..S...AK.RS.KD..IN.IDA.EEQ.AIPPL..|.Q...| 236

QYQNIFGPLVKLEADYDKKLKESQTQDNITVRWDLGLNKKRIAYFTLPKTDSDM/RLMQGDEICLRYKGDLAPLWKGIGH 379
E..RSY...I........Q.....ALEH.S.S.S.A..NRHL.S...STFE.NELKVAI...MI.W.S.MQH.D.E.R.Y 316

VIKVPDNYGDEIAIELR/SSVGAPVEVTHNFQVDFVWKSTSFDRMQSALKTFAVDETSVSGYIYHKLLGHEVEDVITKCQ 458
IVRL.NSFQ.TFTL..KP.KTPP.THL.TGFTAEFI..G..Y....D...K..I.KK.I...L.Y.I...Q.V.ISFDVP 396
                    I                                                  Ia
LPKRFTAQGLPDLNHSQVYAVKTVLQRPLSLIQGPPGTGKTVTSATIVYHLARQGNGPVLVCAPSNIAVDQLTEKIHQTG 538
...E.SIPNFAQ..S..SN..SH.......|..........|.........SKIHKDRI........V...H.AA.LRDL. 476

LKVVRLCAKSREAIDSPVSFLALHNQIRNMDSMPELQKLQQLKDETGELSSADEKRYRALKRTAERELLMNADVICCTCV 618
......T.....DVE.S..N...../LVGRGAKG..KN.LK....V....AS.TK.FVK.V.KT.A.I.NK...V..... 555
            II                                  III
GAGDPRLAKMQFRSILIDESTQATEPECMVPVVLGAKQLILVGDHCQLGPVVMCKKAAKAGLSQSLFERLVVLGIRPIRL 698
....K..DTK/..TV|.......|.S....LI.I.K....V|.....Q.....II|ER...D...K.......IS..HV...| 634
 IV
QVQYRMHPALSAFPSNIFYEGSLQNGVTAADRVKKGFDFQWPQPDKPMFFYVTQGQEEIASSGTSYLNRTEAANVEKITT 778
E....|.N.Y..E...NM..........IEQ.TVPNSKFP..IRGI..M.WANY.R..ISAN...F...I..MNCER.I. 714
                                                V
KLLKAGAKPDQIGIITPYEGQRSYLVQYMQFSGSLHTKLYQEVEIASVDAFQGREKDFIILSCVRANEHQGIGFLNDPRR 858
..FRD.V..E...V........A.IL....MN...DKDL.IK..V|..........Y..|.......Q.A....R.|.. 794
 VI
LNVALTRARYGVIIVGNPKALSKQPLWNHLLNYYKEQKVLVEGPLNNLRESLMQF//SKPRKLVNTINPGARF/MTTAMY 935
...G...K..LV.L..|.RS.ARNT......IHFR.KGC....T.D..QLCTV.LVRPQ...TERPM.AQFNVESEMGDF 874

DAREAIIPGSVYDRSSQGRPSSMYFQTHDQIGMISAGPSHVAAMNIPIPFNLVMPPMPPPGYFGQANGPAAGRGTPKGKT 1015
PKFQDFDAQ.MVSF.GQIGDFGNA.VDNTELSSYINNEYWNFENFKSAFSQKQNRNEIDDRNLY.EEASHLNSNFARELQ 954

GRGGRQKNRFGLPGPSQTNLPNSQASQDVASQPFSQGALTQGYISMSQPSQMSQPGLSQPELSQDSYLGDEFKSQIDVAL 1095
REEQKHELSKDFSNLGI*                                                              971

<-- GACTCCACGTACCAGGGAGAGCGGGCTTACCAGCATGGCGGGGTGACGGGGCTGTCCCAGTATtaaaaggtggc
 S  Q  D  S  T  Y  Q  G  E  R  A  Y  Q  H  G  G  V  T  G  L  S  Q  Y  *          1118

ggcggaagagctaagcaacgtggcttagtccatcagcatcttattctgggtaataaaaaatg                  3602
```

B

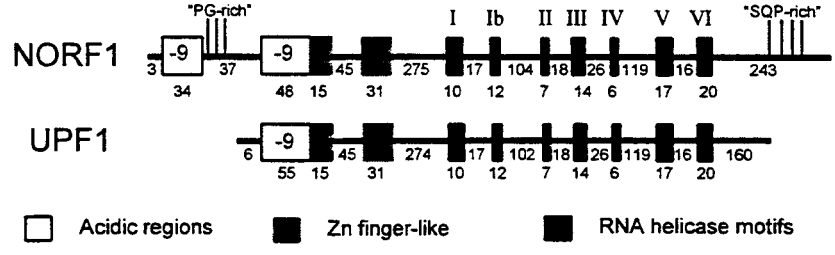


Table 9. --Alignment of Consensus Motifs Shared Between Human NORF1 and Other Eukaryotic Members of the RNA Helicase Superfamily I.

Gene	Species	I	Ib	II	III
hNORF1	human	488 LIQGPPGTGKT 17 VLVCAPSNIAVD 104 ILIDEST 18 ILVGDHCQLGPVVM			
UPF1	*S.cerevisiae*	426 . . . . . 17 I . . . . . V . . 102 V . . . . . 18 . . . . . Q IL			
UPF1-like	*S.pombe*	420 . . . . . 23 . . . . . V . . 104 V . . . . A . 18 V . . . . . Q			
MOV.10	mouse	405 I . F . . . . . 18 I . A . . . . . SGA . 80 IF . . . AG 27 V . A . . PR . . GPVLR			
SEN1	*S.cerevisiae*	1234 . . . . . 41 I . I . . . . . A . . 169 VI . . . AC 18 . M . . PN . . P . T . L			
mSMUBP	mouse	209 I . H . . . . . 16 K . C . . . . . 122 VVV . . CA 17 . . A . . R . . P . TTV			
hSMUBP	human	210 I . H . . . . . 16 . . C . . . . . 122 VV . . . CA 17 . . A . . K . . P . TTV			
DNA-HEL	human	210 I . H . . . . . 16 . . C . . . . . 122 VV . . . CA 17 . . A . . K . . P . TTV			
Ins-Enh	hamster	209 I . H . . . . . 16 I . C . . . . . V . . 122 VVV . . CA 17 . . A . . R . . P . TTI			
CONSENSUS		**UUXGPPGTGKT**	**UUXCAPSNXAXD**	**UUUDEXX**	**UUXGDXXQLXPXXX**
			G		

	IV	V	VI	Accession No.
hNORF1	26 LQVQYR 119 IASVDAFQGREKDFIIL	16 PRRLNVALTRARYGVIIVGN 243		GB U59323
UPF1	26 . E . . . 119 V . . . . . Y . . .	16 . . . . . G . . . . K . . L V . L . . 156		GB M76659
UPF1.like	26 . V . . . 119 V	16 . . . . . K 122		SP W02865
MOV.10	38 . LRN . . 128 VG . . EE . . . Q . RSV . LI	23 . K . F . . V . . . KALLIV . . . 82		GB X52574
SEN1	26 . D . . . 120 FNTI . G . . . Q . . EI . LI	17 F . . M . . . . . KTSIWVL . H 401		GB M74589
mSMUBP	28 . T . . . 117 . K . . G . . . . . EAVL .	16 D . . I . . V . . . . RH . AVICD 381		GB L10075
hSMUBP	28 . T . . . 117 . K . . G . . . . . EAV . .	16 D . . I . . V . . . . RH . AVICD 380		GB L14754
DNA-HEL	28 . T . . . 117 . K . . G . . . . . EAV . .	16 D . . I . . AV . . . RRH . AVICD 380		GB L24544
Ins-Enh	28 . T . . . 117 . K . . G . . . . . EAV . .	16 D . . I . . AV . . . RRH . AVICD 377		GB L15625
CONSENSUS	**LXXXYR**	**UXTU-XFQGXE+XXUUU**	**X+RUNVAUTRA+XXUXUUGX**	
		S	G C	

cysteine-rich region in the human protein extends from codons 123 to 213 (Figure 37.A) and is 63% identical and 90% similar to that of Upf1p (residues 62 to 152). The cysteine-rich region in both the human and Upf1p is preceded by an acidic stretch of 47 and 55 amino acids, respectively, with a net charge of -9. Two motifs within the cysteine-rich region of the human protein (starting at codon 123 and codon 183, respectively), as well as Upf1p (starting at codon 62 and 122, respectively), can be written as $CysX_2CysX_nCysX_3Cys$ (X represents any amino acid and n is 6 for the first and 26 for the second motif). The distances between the two motifs are preserved in both the human protein and Upf1p and the distances between their cysteine-rich and RNA helicase regions differ only by one amino acid residue (Figure 37.A and B). Alternatively, starting with the first cysteine at codon 122, or 62 in Upf1p, another putative zinc finger motif can be written as $CysX_2CysX_{26}HisX_3His$. All three motifs can be partially aligned with the zinc finger/knuckle motif $CysX_{2-5}CysX_{4-12}Cys/HisX_{2-4}Cys/His$, which is found in some zinc finger DNA binding proteins as well as in polypeptides that interact with RNA (Burd and Dreyfuss, 1994).

Because the human protein exhibits the highest degree of homology to the group I RNA helicase Upf1p (Figure 37.A) and contains a very similar overall polypeptide domain structure as Upf1p (Figure 37.B), I conclude that the human protein, which I named NORF1 (nonsense mRNA reducing factor 1), is a putative group I RNA helicase and the first mammalian structural homologue of yeast

Upf1p, a yeast protein responsible for reducing nonsense mRNA levels (Leeds et al., 1991).

Compared to Upf1p, NORF1 has 63 and 83 more amino acids on its N- and carboxy (C) -terminal ends, respectively. These extra tails contain motifs that are absent from yeast Upf1p, such as an additional acidic stretch of 34 amino acids with a net charge of -9 followed by three repeats of a proline/glycine-rich motif (PGGXG) at the N-terminal tail. This glycine-rich region could be involved in RNA binding (Burd and Dryfuss, 1994). There are also multiple SQP and SQ repeats at the C-terminal tail (Figure 37.B) but a structural or functional role for this motif has not been determined.

Expression of *NORF1*

If NORF1 is involved in degrading nonsense mRNA, I would expect that it is expressed in every tissue, because any cell can potentially generate nonsense mRNA, for example via imprecise pre-mRNA splicing. Northern blot analysis was performed using total RNA from several human cell lines representing various human tissues and probed with the human 3.6-kb *NORF1* probe. A predominant ~5.5-kb and a minor ~3.7-kb transcript in all samples (Figure 38), suggesting that *NORF1* is expressed in many tissues.

The size of the 3.7-kb transcripts corresponds very well with the size of the 3.6-kb cDNA clone and, therefore, could represent mature *NORF1* mRNA. The 5.6-kb mRNA might represent an incompletely spliced precursor of the mature 3.7-kb *NORF1* mRNA. If this is true, I would expect that levels of the 5.6-

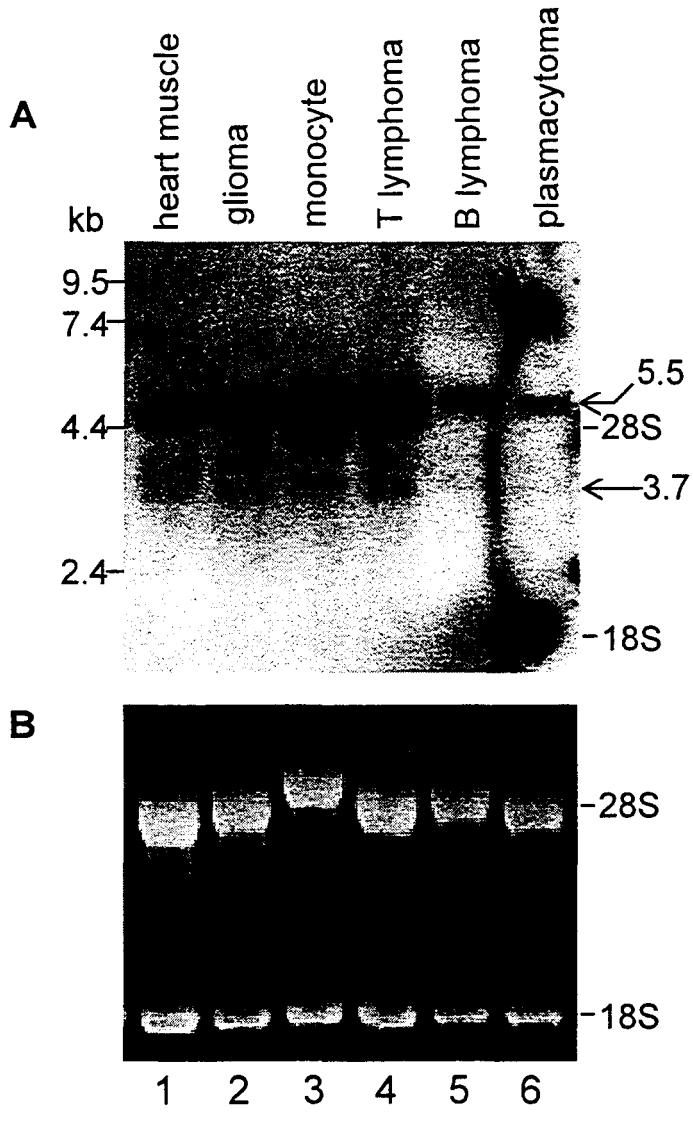


Figure 38. Northern blot analysis of NORF1 mRNA in human cell lines. **(A)** 5 μg of total RNA was subjected to northern blot analysis and hybridized with the complete human clone 3.6 probe. The blot was subjected to flurography for 2 days. RNA size markers are indicated in kilobases (kb) on the left and the position of the 18S and 28S RNA and the two NORF1 transcripts on the right. **(B)** Photograph of the ethidium bromide stained gel. The arrows point to the hybridizing bands at 5.5 and 3.6 kb.

kb transcript would be lower in both total and cytoplasmic total RNA preparations when compared to the signal of the 3.7-kb band. However, as detected by northern blot analysis, levels of the 5.6-kb transcripts are higher in both total and cytoplasmic RNA preparations than levels of the 3.6-kb transcript (compare in Figure 34 lane 4 with lane 3). Therefore, it seems more likely that the two transcripts represent mature mRNA that was generated from a common pre-mRNA by either differential splicing or differential usage of polyadenylation sites. To isolate a cDNA clone that might represent the 5.5-kb transcript, I screened a human Jurkat λ-phage cDNA library with a 5' probe of clone 3.6 (*Xho*I-*Sal*I fragment in Figure 39). I identified 22 clones; eight of these clones were plaque purified and plasmids were excised from plaque-purified phage. Restriction enzyme digestion of plasmids prepared from the 8 clones (3.1.1.1/2, 3.2.1.1/2, 9.1.1.1/2, 15.1.1.1/2, 17.1.1.1/2, 19.1.1.1/2, 21.1.1.1/2, 27.1.1.1/2) identified inserts of different sizes (Appendix Figure 2). The largest insert (clone 15.1.1/2) was 5.5-kb in length. This clone, whose size corresponds very well with that of the 5.5-kb transcript, was named clone 5.5 and used for further analyses. Restriction mapping analysis (Appendix Figure 3.A, 3.B) and partial DNA sequencing of clone 5.5 revealed that clone 3.6 overlaps with clone 5.5 . For example, I identified in clone 5.5 all restriction enzyme sites that were found in clone 3.6 (Figure 39). Moreover, I verified in clone 5.5 sequences of clone 3.6 that contain the translational start and stop sites and the polyadenylation signal site AATAAA (Figure 39). I also detected at the 3' end of clone 5.5 a second

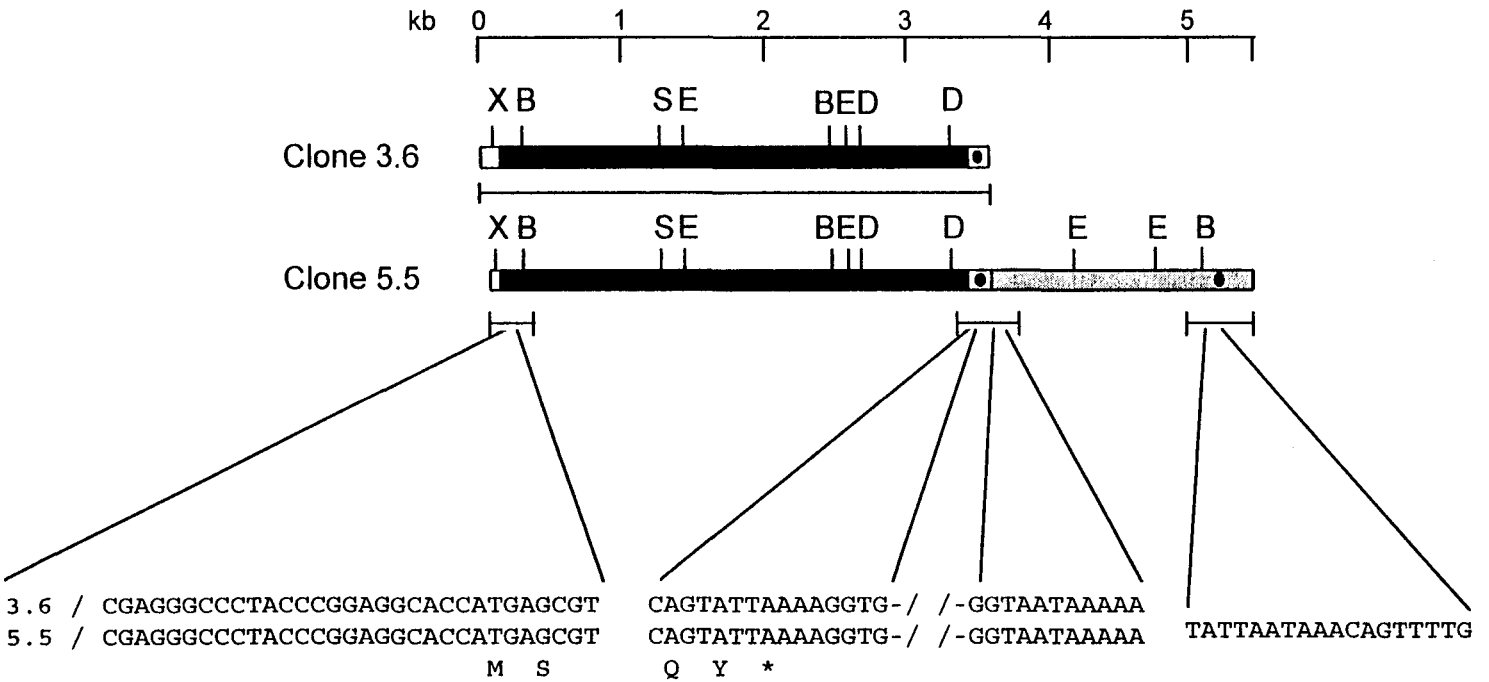


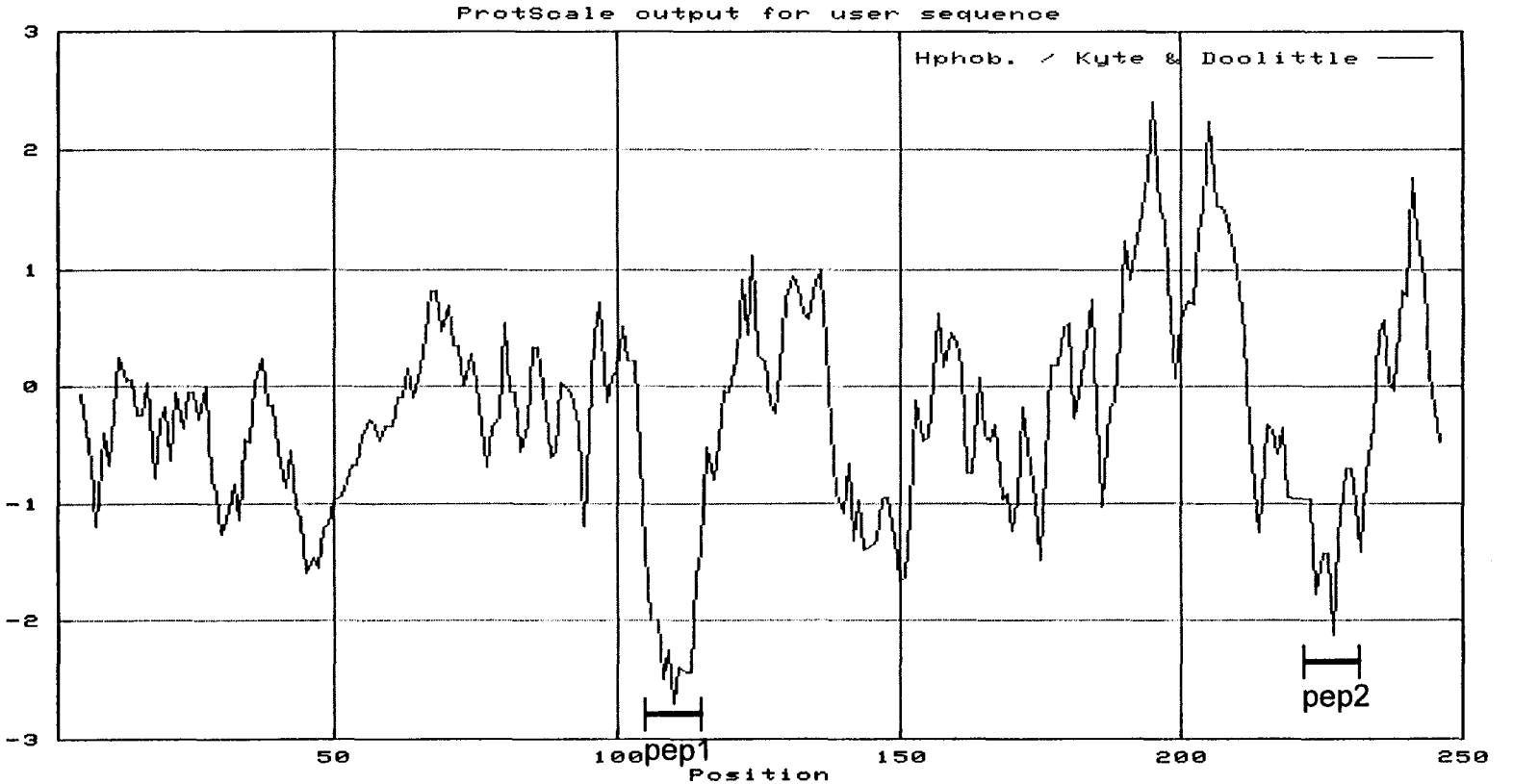
Figure. 39. Schematic representation and partial sequence alignment of the human NORF1 clones 3.6 and 5.5. ORFs are indicated by black boxes, untranslated regions by white and gray-shaded boxes, and poly(A) addition signal sites by black circles. Sequenced regions are underlined. Restriction cutting sites are X, XhoI; B; BstXI; S, SalI; E, Eco47III; D, DraI. Relative size of the two clones can be seen by comparison to the scale above. Sequenced regions of each gene are shown by blunt end bars below each gene. Key regions of the NORF1 gene nucleotide sequence identical between the two clones are shown below each gene except for the additional poly(A) addition signal site in clone 5.5.

classical polyadenylation signal site (AATAAA). I, therefore, propose that both the 3.7-kb and 5.5-kb transcript contain the same ORF and differ in their 3' untranslated region. Thus, the two transcripts might have been generated from the same pre-mRNA by differential polyadenylation signal site usage. However, I cannot completely exclude that clone 3.6 represents a truncated cDNA fragment of the 5.6-kb transcript. It may be possible that the 3.6-kb transcript is encoded by a gene that is different from *NORF1* but shares with *NORF1* some DNA sequence identity.

Expression of NORF1 Protein

To determine if a NORF1-like protein of about 130-kDa is present within human cells, I generated a rabbit antiserum reactive against peptides deduced from hydrophilic regions of the putative ORF of *NORF1* (underlined in Figure 40). The synthesized peptides (NORF1/pep#1 and #2) were coupled to keyhole limpet hemocyanin (KLH) and used to immunize rabbits. The antibodies were affinity-purified from serum on the respective NORF1-peptide columns and used in western blot analysis. The affinity purified anti-NORF1-pep#1 antibodies stained on a western blot the radiolabeled 130-kDa protein that was *in vitro* translated from a clone 3.6 template (Figure 41). This antibody also detected a 130-kDa protein in both mouse (lane 3) and human cells (lane 4). This suggests that the ORF of *NORF1* can be translated *in vitro*, and *in vivo*, into a 130-kDa polypeptide. It is not known if the anti-NORF1-pep#1 antibodies detect yeast Upf1p but it is unlikely that it would do so as the peptide epitope used to

Figure40. Hydrophobicity plot of NORF1 used to design NORF1 peptides. The plot was generated by submitting the polypeptide sequence below the hydrophobicity plot to the ExPASy molecular biology server at http://expasy.hcuge.ch/www/ tools.html. The bracket in the plot represents the region of NORF1 residues used as an immunogen. The exact residues are shown below the plot and are in bold and underlined.



10 20 30 40 50 60 70
MSVEAYGPSS QTLTFLDTEE AELLGADTQG SEFEFTDFTL PSQTQTPPGG PGGPGGGGAG SPGGAGAGAA
80 90 100 110 120 130 140
AGQLDAQVGP EGILQNGAVD DSVAKTSQLL AELNF**EEDEE DTYYTKDLPI HAC**SYCGIHD PACVVYCNTS
150 160 170 180 190 200 210
KKWFCNGRGN TSGSHIVNHL VRAKCKEVTL HKDGPLGETV LECYNCGCRN VFLLGFIPAK ADSVVVLLCR
220 230 240 250
QPC**ASQSSLK DINWDSSQWQ PLIQDRC**FLS WLVKIPSEQE

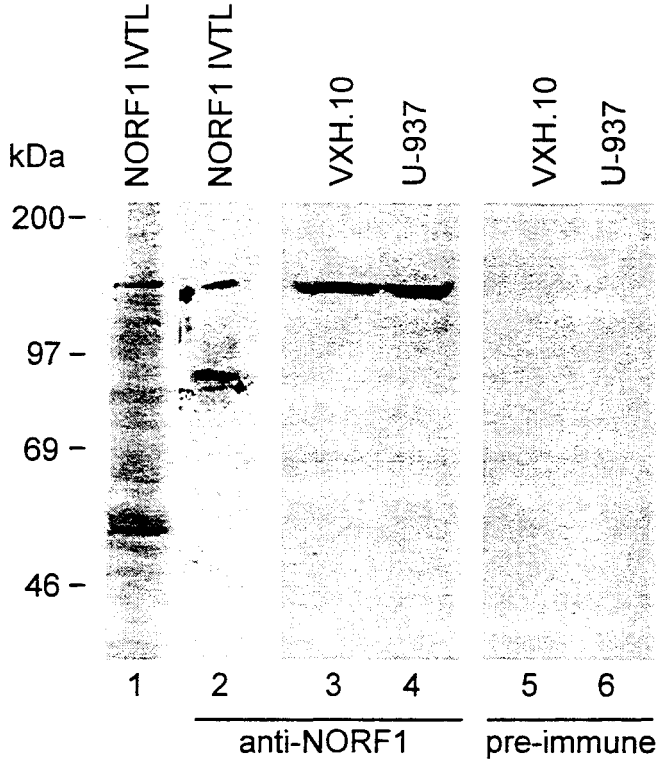


Figure 41. *In vitro* transcription-translation (IVTL) and western blot analysis of IVTL products and cellular lysates. Western blot analysis of IVTL products (lane 2), and analysis of cell extracts from mouse VXH (lanes 3 and 5) and human U-937 cells (lanes 4 and 6) with rabbit anti-NORF1-pep#1 peptide antibodies (lanes 2-4) and preimmune serum (lanes 5 and 6). Whole IVTL reaction product (lane 1) (5 μl) was detected by fluorography for 4 days. Molecular mass protein standards are indicated in kilodaltons (kDa) on the left.

generate the anti-NORF1-pep#1 antibodies is not found within the predicted *UPF1* polypeptide. Based on these data, I conclude that I have cloned a human cDNA (*NORF1*) that exhibits a large degree of amino acid identity to that of yeast Upf1p, a group 1 RNA helicase required for nonsense codon-mediated mRNA degradation. It also suggests that both human and mouse cells express the NORF1 protein.

Functional Analysis of NORF1

NORF1 Complementation of an *upf1* Null Yeast Strain

To determine if NORF1 is capable of reducing nonsense mRNA levels in a *upf1* null (*upf1)* yeast strain, I transformed the yeast strain PLY154 *ura3::HIS4* (PLY154) with a plasmid that contains the complete ORF of *NORF1* cDNA under the control of the inducible *gal1-10* promoter. PLY154 cells contain a nonsense *LEU2* allele (*leu2-1)*, which encodes a nonsense *leu2-1* mRNA. Because Upf1p is absent from PLY154 cells, nonsense *leu2-1* mRNA becomes more stable and a low degree of translational readthrough can generate enough Leu2p to support the growth of PLY154 cells in the absence of leucine. If NORF1 can replace the function of Upf1p in yeast, *leu2-1* mRNA should rapidly be degraded and the transformants will not grow in the absence of leucine. Six transformants were isolated and tested for their inability to grow in the absence and presence of leucine. The growth of these transformants was compared to yeast cells transformed with a control plasmid lacking an insert (pYes 2.0, Invitrogen).

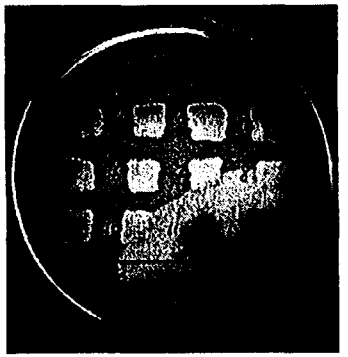
Figure 42 shows the results from these experiments. Yeast containing either the *NORF1* cDNA (a-d) or the control plasmid (e-h) were both able to grow in the absence of uracil (indicating the presence of the transformed plasmid), the presence of galactose (which induces expression of the *NORF1* cDNA), and in the absence of leucine, indicating that levels of nonsense *leu2-1* mRNA have not been decreased. Therefore, I conclude that the presence of the *NORF1* cDNA under inducing conditions is not able to replace the function of Upf1p, that is, to induce the degradation of nonsense mRNA.

Leeds and co-workers (1992) were able to determine that mutations in the RNA helicase region of the *UPF1* gene led to a dominant-negative effect in yeast cells containing a wild type copy of the *UPF1* gene. Based on these observations, it may be possible NORF1 rather blocks Upf1p leading to a *UPF1*-minus (*upf1)* phenotype. To test this idea, yeast cells containing a wild type *UPF1* gene were transformed with the plasmid containing the complete *NORF1* ORF or with a control plasmid that does not contain the *NORF1* ORF. If NORF1 acts in a dominant-negative fashion, it will interfere with Upf1p and prevent the degradation of the nonsense mRNA transcribed from the *leu2-1* gene, which will change the phenotype of the transformed yeast cell from Leu^- to Leu^+. Transformants were generated and grown in the absence of uracil (which selects for the presence of the transformed plasmid library) and the presence of galactose (which induces the expression of the NORF1). However, when replica plated on plates with or without leucine, none of the cells transformed with the *NORF1* (Figure 43 a-d) or control plasmid (e-h) grew on plates

Figure 42. Complementation of a UPF1 null (*upf1*) yeast strain with the NORF1 cDNA. The yeast strain PLY154 *ura3::HIS4* was transformed with the galactose inducible NORF1 cDNA yeast shuttle plasmid (11.2.2) or with the galactose inducible yeast shuttle plasmid pYes 2.0 with no insert. Transformants were patched onto plates lacking uracil (Ura^-) and replica plated to the indicated plates. The plasmid used to transform the yeast is shown above the plate and the composition of the plate medium is shown below the plate. Whether cell growth was observed is noted in parentheses after the medium.

NORF1

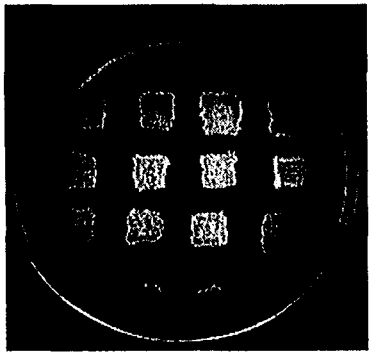
a



ura- (Growth)

NORF1

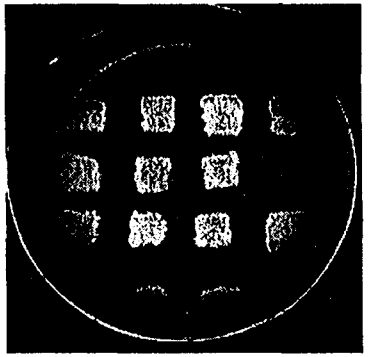
b



ura- / leu- (Growth)

NORF1

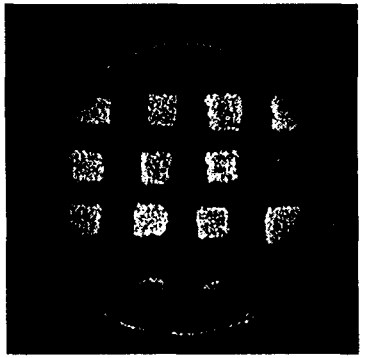
c



ura- / gal+ (Growth)

NORF1

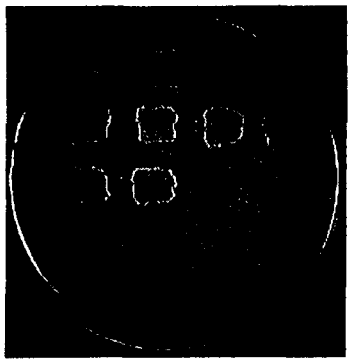
d



ura- / gal+ / leu- (Growth)

pYes 2.0

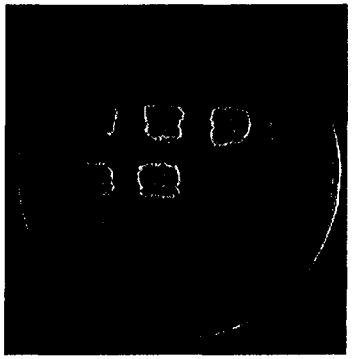
e



ura- (Growth)

pYes 2.0

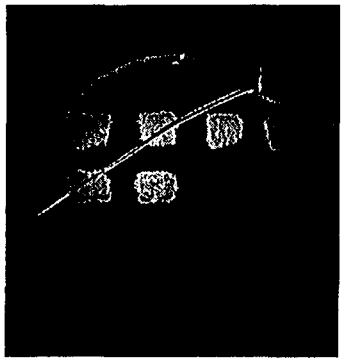
f



ura- / leu- (Growth)

pYes 2.0

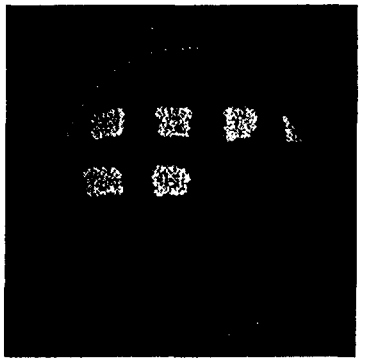
g



ura- / gal+ (Growth)

pYes 2.0

h

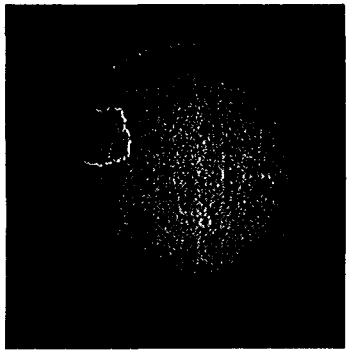


ura- / gal+ / leu- (Growth)

Figure 43. Dominant-negative test of a UPF1 null (*upf1*) yeast strain with the NORF1 cDNA. The yeast strain SL988-11A was transformed with the galactose inducible NORF1 cDNA yeast shuttle plasmid (11.2.2) or with the galactose inducible yeast shuttle plasmid pYes 2.0 with no insert. Transformants were patched onto plates lacking uracil (Ura^-) and replica plated to the indicated plates. The plasmid used to transform the yeast is shown above the plate and the composition of the plate medium is shown below the plate. Whether cell growth was observed is noted in parentheses after the medium.

NORF1

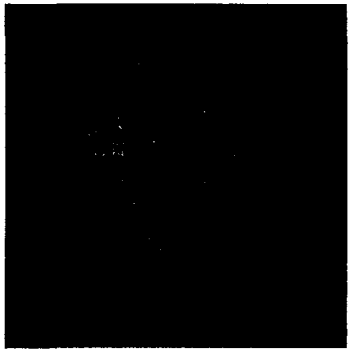
a



ura- (Growth)

NORF1

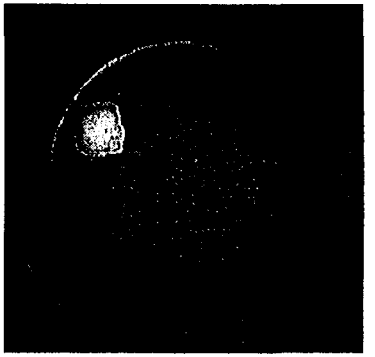
b



ura- / leu- (No Growth)

NORF1

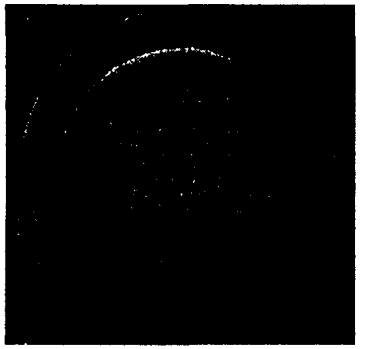
c



ura- / gal+ (Growth)

NORF1

d



ura- / gal+ / leu- (No Growth)

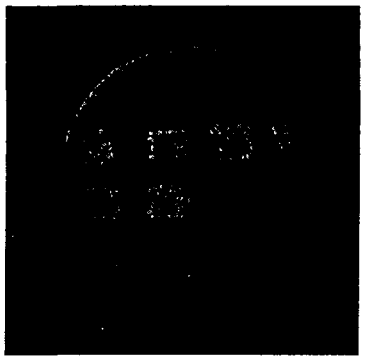
pYes 2.0

e

ura- (Growth)

pYes 2.0

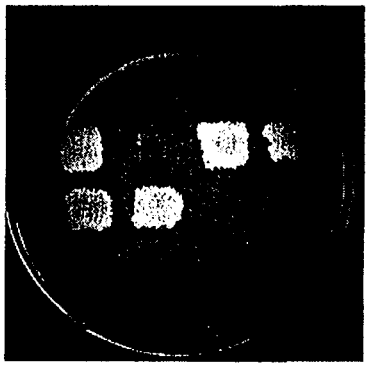
f



ura- / leu- (No Growth)

pYes 2.0

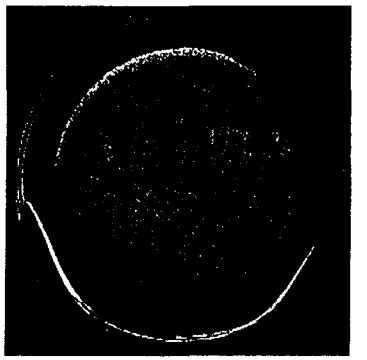
g



ura- / gal+ (Growth)

pYes 2.0

h



ura- / gal+ / leu- (No Growth)

supplemented with leucine. From this result I conclude that NORF1 does not block the function of Upf1p in yeast cells. But in fact, more control experiments need to be done to confirm the expression of NORF1 protein in the yeast cells grown under inducing conditions.

In summary, NORF1 when expressed in yeast cells does not replace nor block the function of Upf1p. However, it is possible that in both the complementation and dominant-negative approaches NORF1 was expressed at levels that were not high enough to replace Upf1p or to act dominant-negative. Alternatively, NORF1 might not replace the function of Upf1p in yeast cells, because it cannot interact with another protein that is required to induce the degradation of nonsense mRNA.

NORF1 Antisense RNA Expression in Mammalian Cells

To determine if the NORF1 protein controls nonsense mRNA levels in a mammalian cell, I stably expressed *NORF1* antisense transcripts from a transfected *NORF1* cDNA in mouse B cells containing a immunoglobulin μ mRNA with a nonsense codon (Jäck and Wabl, 1989) (Figure 44). If NORF1 is involved in the regulation of nonsense mRNA, downregulation of NORF1 synthesis by antisense *NORF1* transcripts should result in an increase in μ mRNA levels.

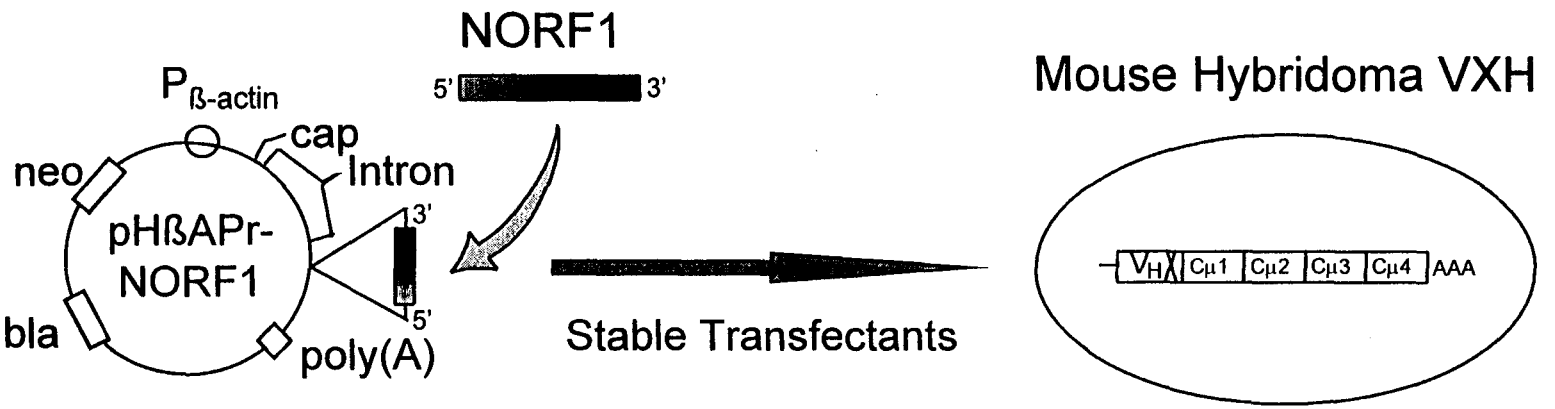


Figure 44. Schematic representation of the antisense orientation cloning of NORF1 into the expression vector pHβ-APr-1-neo and subsequent transfection into the mouse B cell hybridoma VXH. The complete NORF1 ORF was excised on a XhoI/HindIII fragment and cloned into the vector prepared with HindIII/BamHI. The pHβ-APr-1-neo contains a β-actin promoter (P), a 5' UTR intron, a SV40 poly(A) addition site, an ampicillin resistance gene (bla), and a neomycin/G418 resistance gene (neo). Structure of the nonsense μ mRNA in VXH is shown in the cell. The exons are indicated by boxes (V_H variable; C, constant) and the length of translation products by lines below the mRNA. The position of the nonsense codon at the end of the VH exon is represented by the X.

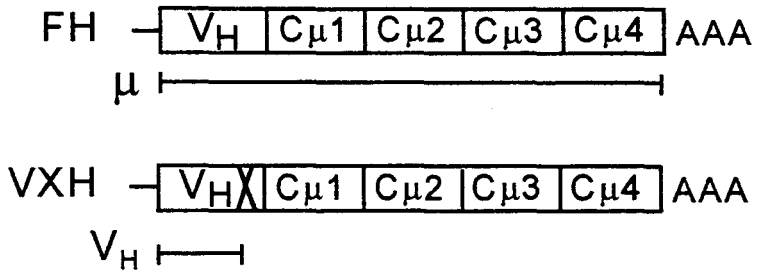
Construction of a Mammalian Antisense *NORF1* Expression Vector

The complete *NORF1* ORF was cloned into the mammalian antisense expression vector pHβApr-1-neo (Gunning et al., 1987). The *NORF1* ORF was excised from plasmid pGem7Z-*NORF1*(3.6-kb) as a 3.5-kb *Hind*III/*Xho*I fragment and cloned in a two-step ligation procedure into the expression vector prepared with *Hind*III/*Bam*HI.

Stable Expression of *NORF1* Antisense RNA in Mouse Cells

To determine whether NORF1 is important for controlling levels of nonsense mRNA in mammalian cells, I introduced the mammalian antisense *NORF1* expression vector into the mouse hybridoma line VXH. VXH transcribes an endogenous μ gene with a TAG nonsense codon in its variable (V_H) gene exon (Figure 45). The nonsense mutation reduces the level of μ mRNA in VXH by a factor of ~15 when compared to that of functional μ mRNA in the hybridoma line FH (Jäck and Wabl, 1989). FH cells contain the same μ gene as VXH cells except that the TAG nonsense codon in the V exon is changed to TAC (Jäck and Wabl, 1989). When I analyzed VXH transfectants on western blots, I found that antisense clone VXH.3 produced less NORF1 protein (Figure 46A, lane 1) than control vector-transfected VXH (lane 3) and untransfected FH cells (lane 4). However, transfected and untransfected VXH clones produce about the same amount of the heavy chain binding protein, BiP (Haas, 1994). This suggests that human *NORF1* antisense transcripts downregulated the expression of mouse

A



B

V_H81X with Leader to the VXH nonsense codon

MDFGLSLVFLVLILKSGVQCEVQLVESGGGLVQPRESLKLSCESNEYEFPSHDMSWVRKTPEKR
LELVAAINSDGGSTYYPDTMERRFIISRDNTKKTLYLQMSSLRSEDTALYYCARHD

Theoretical Mw for the protein sequence
MDFGLSLVFL ... TALYYCARHD : Theoretical Mw: **13691.44**

C

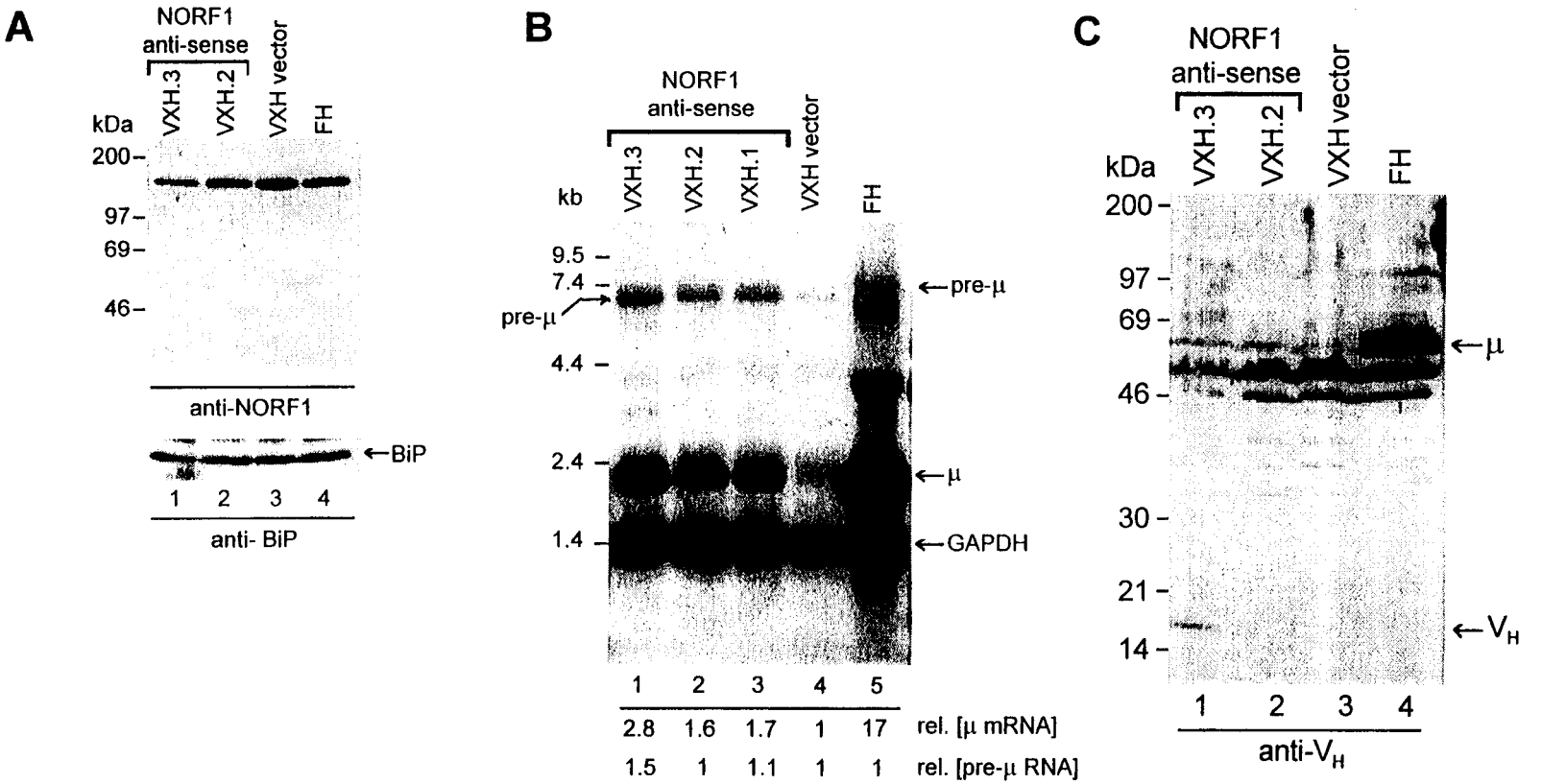
V_H81X without Leader to the VXH nonsense codon

GVQCEVQLVESGGGLVQPRESLKLSCESNEYEFPSHDMSWVRKTPEKRLELVAAINSDGGSTYY
PDTMERRFIISRDNTKKTLYLQMSSLRSEDTALYYCARHD

Theoretical Mw for the protein sequence
GVQCEVQLVE ... TALYYCARHD : Theoretical Mw: **11914.23**

Figure 45. Schematic diagram and sequence of the two predicted polypeptides that can be translated from the μ immunoglobulin nonsense mRNA in VXH cells. **(A)** Structures of functional μ mRNA in FH cells and nonsense μ mRNA in VXH cells. The exons are indicated by boxes (V_H variable; C, constant) and the length of translation products by lines below the mRNA. The position of the TAG nonsense codon at the end of the V_H exon, is represented by the X. The m gene in VXH differs from that in the FH cell by the presence of the TAG stop codon. **(B)** Sequence of the polypeptide translated from nonsense mRNA in VXH cells before cleavage of the leader sequence. **(C)** Sequence of the polypeptide after cleavage of the leader sequence. The predicted molecular mass of each polypeptide is shown below each sequence in bold letters. The nucleotide sequences used to caculate the molecular mass were obtained from GenBank entries: accession #X01113, and #K02890 and from Wabl et al., (1985).

Figure 46. Effect of human *NORF1* antisense RNA expression on μ mRNA levels in transfected mouse cells. **(A)** Western blot analysis of mouse NORF1 in untransfected FH cells (lane 4) and VXH cells stably transfected with the human *NORF1* antisense expression vector (lanes 1 and 2) and the expression vector alone (lane 3). The blot was first probed with anti-NORF1-pep1 antibodies (see Figure X) and then, to control for loading, restained with anti-BiP antibodies. **(B)** Northern blot analysis of μ mRNA expression in transfected VXH and untransfected FH cells. 5 μg total RNA was analyzed as described in Figure 17. The blot was probed with a mouse Cμ and a rabbit GAPDH cDNA probe. The positions of m and GAPDH mRNAs are indicated on the right. **(C)** Western blot analysis of polypeptides that react with anti-V_H peptide antibodies in transfected VXH and untransfected VXH cells. The blot was probed with a rabbit anti-V_H peptide antiserum that reacts with an epitope encoded by the V_H region in FH and VXH cells (Hartwell & Jäck, unpublished observations). The positions of the V_H and full length μ polypeptides are indicated on the right. Bands other than those indicated by arrows are also detected in cells that do not produce any Ig chains and thus, result from reactions of rabbit antibodies with non-Ig polypeptides. To control for loading, the blot was reprobed with a rabbit anti-BiP serum and similar signals of a single 70-kDa band (BiP) were observed in all 4 lanes.


A
NORF1
anti-sense
VXH.3
VXH.2
VXH vector
FH
kDa
200
97
69
46
anti-NORF1
←BiP
1 2 3 4
anti- BiP
B
NORF1
anti-sense
VXH.3
VXH.2
VXH.1
VXH vector
FH
kb
9.5
7.4
pre-μ
4.4
2.4
1.4
←pre-μ
←μ
←GAPDH
1 2 3 4 5
2.8 1.6 1.7 1 17 rel. [μ mRNA]
1.5 1 1.1 1 1 rel. [pre-μ RNA]
C
NORF1
anti-sense
VXH.3
VXH.2
VXH vector
FH
kDa
200
97
69
46
30
21
14
←μ
←V_H
1 2 3 4
anti-V_H

NORF1 protein in antisense clone VXH.3. The downregulation of NORF1 protein correlated with an increase in nonsense μ mRNA in VXH.3 transfectants. On northern blots, I found about 3 times more nonsense μ mRNA in antisense clone VXH.3 (Figure 46B, lane 1) than in a VXH clone transfected with the expression vector alone (lane 4) (Table 10). One possibility to explain why the level of nonsense μ mRNA was not restored to that of functional μ mRNA in FH cells (Figure 46B, lane 5) is that I still detected some NORF1 protein in antisense clone VXH.3 (Figure 46A, lane 1). Because the levels of nonsense μ mRNA are increased in VXH.3 transfectants, I should also detect a shorter Ig chain that is encoded by the ORF extending from the start codon to the nonsense codon μ mRNA (Figure 45.B and C and Figure 46.C). Indeed, I detected a band of approximately 15 kDa by western with anti-V_H antibodies in antisense clone VXH.3 (Figure 46, lane 1), but not in vector-transfected VXH (lane 3) and wild-type FH cells (lane 4). I conclude that the transfection of an antisense NORF1 gene into VXH cells resulted in increased levels of nonsense μ mRNA and, therefore, that NORF1 is responsible for controlling nonsense μ mRNA levels. However, this conclusion is based on the result obtained with this one VXH transfectant as well as from one western blot and northern blot of the indicated transfectants. The accuracy of the data obtained needs to be confirmed by repeating the western and northern blot analysis on these and possibly other independent transfectants.

Table 10. -- Quantitation of Northern Blot Analysis in Figure 46[a]

Cell line and transfectant clone number	RNA loaded	μ	pre-μ	GAPDH	rel [μ] [μ/GAPDH]	rel [pre-μ] [pre-μ/GADPH]
		[CPM]	[CPM]	[CPM]		
FH	5 μg	289.5	11.1	132.3	17	1
VXH vector	5 μg	7.5	3.9	56.9	1	1
VXH.1	5 μg	22.5	8.0	101.2	1.7	1.1
VXH.2	5 μg	22.6	8.2	109.9	1.6	1
VXH.3	5 μg	35.3	10.7	99.3	2.8	1.5

[a]Radioactivity of respective bands in each Northern blot (CPM) was determined by using a betascope blot analyzer. Relative steady-state level of respective RNA was calculated by dividing CPM of μ by that of GAPDH in the same lane. Blots were determined to be free of background by emperical observation so no adjustments of the numbers collected were made.

% [μ] is the percent of [μ] relative to that of the hybridoma VXH by setting VXH value equal to 100%.

An [*] indicates the absence of data due to either background hybridization or to the absence of this data to perform caculations.

Note: This data is the result of only one experiment.

Isolation of a Mouse *NORF1* Homologue

The current work in our lab involves the analysis of immunoglobulin mRNA with nonsense codons in murine B cells. To study the function of the *NORF1* gene in mouse cells, the mouse *NORF1* cDNA was isolated. Southern blot analysis of mouse genomic DNA using the complete human *NORF1* cDNA revealed the presence of hybridizing bands under high stringent conditions (data not shown). I concluded from this result that the mouse genome likely encodes a gene that has nucleotide identity with the human *NORF1* gene. Therefore I used the human 3.6-kb *NORF1* cDNA probe to screen approximately 2 X 10^5 individual cDNA plaques from a mouse C2 muscle cell line oligo-d(T)-primed λ-phage library under low stringency hybridization conditions. The library was generated from a mouse muscle cell line and, because *NORF1* is likely to be expressed in all cells, the library should contain *NORF1* cDNA clones. Two phage clones were identified that hybridized with the human 3.6-kb *NORF1* probe. One of these clones was plaque purified using low stringency hybridization, the plasmid was excised from the phage, and was found to contain a 4.5-kb cDNA insert (Appendix Figure 4.A). The plasmid and insert were also mapped (Appendix Figure 4.B, C, D, E, F, and G). Northern blot analysis using the 4.5-kb mouse insert revealed a single band of about 4.6-kb, the size expected for a full-length cDNA clone of NORF1 (Figure 47).

The nucleotide sequence of the insert was partially determined and was very similar at the nt and aa level to that of the corresponding human sequence.

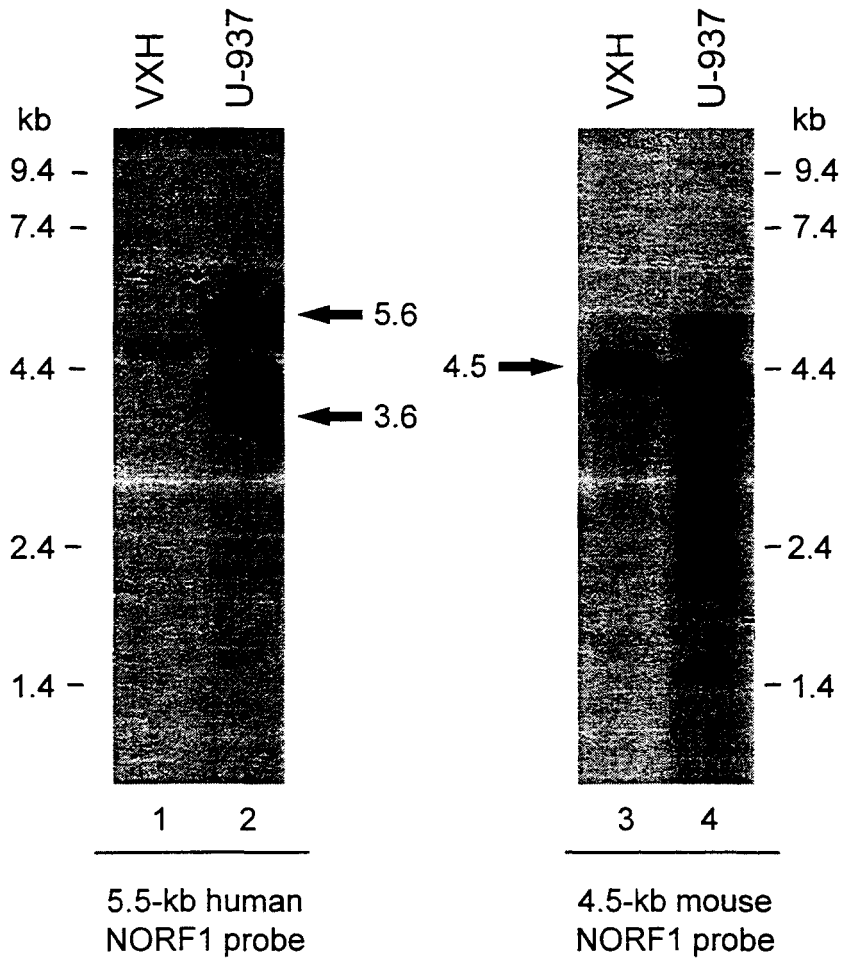
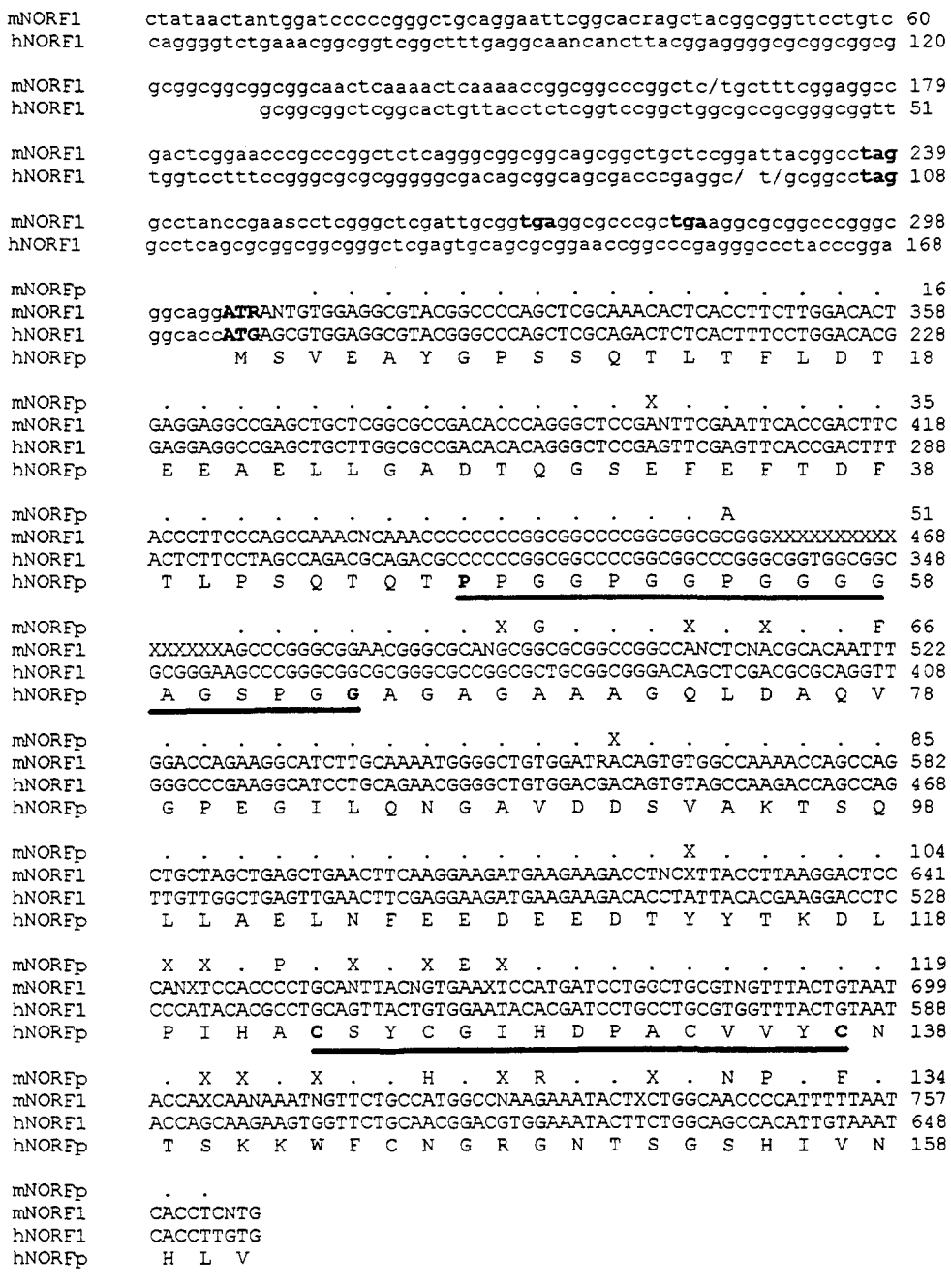


Figure 47. Northern blot analysis of total human and mouse RNA using human and mouse NORF1 probes. 10 μg of total RNA was separated by formaldahyde gel electrophoresis and blotted. Each blot was probed with the indicated NORF1 probe onto GeneScreen Plus. Two strips with identical RNA samples were cut from the membrane. One was hybridized with the 4.5-kb mouse NORF1 and the other with the 5.5-kb human NORF1. RNA size markers are indicated in kilobases (kb) on the left and the right. The position of the two NORF1 transcripts in the center. The blots were exposed to X-ray film for 36 hours at -70°C using an enhancing screen.

(Figure 48). From these sequence data I conclude that I cloned a murine cDNA that exhibits a high degree of identity to the human 3.6-kb *NORF1* gene.

FIGURE 48. Sequence comparison of 5' sequences of human (h) and mouse (m) *NORF1*. The complementary 5' nucleotide and deduced amino acid sequences of *hNORF1* were aligned manually with the 5' nucleotide and deduced amino acid sequence of the mouse 4.5-kb cDNA fragment. The numbers at the right indicate nucleotide and amino acid positions. Untranslated regions are shown in lowercase and translated regions in uppercase letters. The start codon of *NORF1* and the in-frame stop codons upstream of the start codon are in bold. The deduced amino acid sequences of hNORF1 and mNORF1 (in single letter IUPC code) are shown above and below their corresponding partial nucleotide sequences. In the alignments, a period indicates an identical amino acid residue, a X an unidentified nucleic and amino acid residue, and a forward slash a gap of one amino acid or nucleotide introduced for optimal alignments. The cysteine-rich putative zinc finger motifs and the GP-rich motifs are underlined in bold.


mNORF1 ctataactantggatccccgggctgcaggaattcggcacragctacggcggttcctgtc 60
hNORF1 caggggtctgaaacggcggtcggctttgaggcaancancttacggaggggcgcggcggcg 120
mNORF1 gcggcggcggcggcaactcaaaactcaaaaccggcggcccggctc/tgctttcggaggcc 179
hNORF1 gcggcggctcggcactgttacctctcggtccggctggcgccgcgggcggtt 51
mNORF1 gactcggaacccgcccggctctcagggcggcggcagcggctgctccggattacggcctag 239
hNORF1 tggtcctttccgggcgcgcgggggcgacagcggcagcgacccgaggc/ t/gcggcctag 108
mNORF1 gcctanccgaascctcgggctcgattgcggtgaggcgcccgctgaaggcgcggcccgggc 298
hNORF1 gcctcagcgcggcggcgggctcgagtgcagcgcggaaccggcccgagggccctacccgga 168
mNORFp 16
mNORF1 ggcaggATRANTGTGGAGGCGTACGGCCCCAGCTCGCAAACACTCACCTTCTTGGACACT 358
hNORF1 ggcaccATGAGCGTGGAGGCGTACGGGCCCAGCTCGCAGACTCTCACTTTCCTGGACACG 228
hNORFp M S V E A Y G P S S Q T L T F L D T 18
mNORFp . . . . . X 35
mNORF1 GAGGAGGCCGAGCTGCTCGGCGCCGACACCCAGGGCTCCGANTTCGAATTCACCGACTTC 418
hNORF1 GAGGAGGCCGAGCTGCTTGGCGCCGACACACAGGGCTCCGAGTTCGAGTTCACCGACTTT 288
hNORFp E E A E L L G A D T Q G S E F E F T D F 38
mNORFp . . . . . A 51
mNORF1 ACCCTTCCCAGCCAAACNCAAACCCCCCCCGGCGGCCCCGGCGGCGCGGGXXXXXXXXXX 468
hNORF1 ACTCTTCCTAGCCAGACGCAGACGCCCCCCGGCGGCCCCGGCGGCCCGGGCGGTGGCGGC 348
hNORFp T L P S Q T Q T P P G G P G G P G G G G 58
mNORFp . . . . . X G . . . X . X . . F 66
mNORF1 XXXXXXAGCCCGGGCGGAACGGGCGCANGCGGCGCGGCCGGCCANCTCNACGCACAATTT 522
hNORF1 GCGGGAAGCCCGGGCGGCGCGGGCGCCGGCGCTGCGGCGGGACAGCTCGACGCGCAGGTT 408
hNORFp A G S P G G A G A G A A A G Q L D A Q V 78
mNORFp . . . . . X 85
mNORF1 GGACCAGAAGGCATCTTGCAAAATGGGGCTGTGGATRACAGTGTGGCCAAAACCAGCCAG 582
hNORF1 GGGCCCGAAGGCATCCTGCAGAACGGGGCTGTGGACGACAGTGTAGCCAAGACCAGCCAG 468
hNORFp G P E G I L Q N G A V D D S V A K T S Q 98
mNORFp . . . . . X 104
mNORF1 CTGCTAGCTGAGCTGAACTTCAAGGAAGATGAAGAAGACCTNCXTTACCTTAAGGACTCC 641
hNORF1 TTGTTGGCTGAGTTGAACTTCGAGGAAGATGAAGAAGACACCTATTACACGAAGGACCTC 528
hNORFp L L A E L N F E E D E E D T Y Y T K D L 118
mNORFp X X . P . X . X E X 119
mNORF1 CANXTCCACCCCTGCANTTACNGTGAAXTCCATGATCCTGGCTGCGTNGTTTACTGTAAT 699
hNORF1 CCCATACACGCCTGCAGTTACTGTGGAATACACGATCCTGCCTGCGTGGTTTACTGTAAT 588
hNORFp P I H A C S Y C G I H D P A C V V Y C N 138
mNORFp . X X . X . . H . X R . . X . N P . F . 134
mNORF1 ACCAXCAANAAATNGTTCTGCCATGGCCNAAGAAATACTXCTGGCAACCCCATTTTTAAT 757
hNORF1 ACCAGCAAGAAGTGGTTCTGCAACGGACGTGGAAATACTTCTGGCAGCCACATTGTAAAT 648
hNORFp T S K K W F C N G R G N T S G S H I V N 158
mNORFp . .
mNORF1 CACCTCNTG
hNORF1 CACCTTGTG
hNORFp H L V

CHAPTER V

DISCUSSION

The yeast *UPF1* gene plays a defined role in the accelerated degradation of yeast nonsense mRNAs (Leeds et al., 1992; He et al., 1993). The identification of the *UPF1* homologue, NORF1, in both human and mouse cells suggests that mammalian genes reduce levels of nonsense mRNA, at least in part, by similar mechanisms as yeast. Two results support the hypothesis that NORF1 is a *UPF1* homologue. First, the conservation of key functional elements of Upf1p such as the zinc-finger-like domain and RNA helicase region in NORF1 suggest that it may act in a similar manner. Second, antisense NORF1 expression partially reduces, the levels of nonsense mRNA in mammalian cells.

Putative Functions of Motifs Conserved Between UPF1 and NORF1

The Zinc-finger Motif

NORF1 and Upf1p contain very similar cysteine-rich regions which are characteristic of proteins that interact with nucleic acids. The cysteine-rich

domain contains two zinc-finger-like motifs that can be partially aligned with the zinc finger/knuckle motif, $CysX_{2-5}CysX_{4-12}Cys/HisX_{2-4}Cys/His$. Zinc finger/ knuckle motifs are found in some zinc finger DNA binding proteins as well as in polypeptides that interact with RNA (Burd and Dreyfuss, 1994).

The RNA Helicase Domain

NORF1 and Upf1p also contain a very similar RNA helicase domain that is characteristic of RNA helicases. These enzymes can unwind double stranded RNA (reviewed in Schmid and Linder, 1992). Purified Upf1p is able to unwind dsRNA in *in vitro* tests in an ATP-dependent fashion (Czaplinski et al., 1995). Because NORF1 contains a conserved superfamily I ATP-dependent RNA helicase region, it is possible that NORF1 is also able to unwind dsRNA and uses ATP as the energy source. How this activity may affect nonsense mRNA is discussed below.

The Acidic Region

NORF1 and Upf1p contain a stretch of acidic amino acids without conserved residues and NORF1 has an additional stretch with the same net charge as the acidic stretch shared by Upf1p and NORF1. Some DNA-binding proteins require these domains to allow transcriptional activation (Umesono and Evans, 1989). This region, as well as regions discussed below, could bind to other proteins that are part of the machinery that recognize nonsense mRNA.

Possible Functions of Other Motifs in NORF1

The predicted NORF1 protein contains at its N- terminal end a proline-glycine rich region motif that is not present in Upf1p. When DNA databases were searched with the proline-glycine rich region of the NORF1 gene, matches were found with transcription factors that contained proline-glycine rich regions at their N-terminal ends. When these proline-glycine rich regions are changed, the activity of a transcription factor can decrease or is completely abolished (Umesono and Evans, 1989). It is thought that this region is important for interactions of transcription factors with DNA. In NORF1 this region could interact with double stranded RNA.

NORF1 contains a number of SQ-space (serine and glutamine) and SQP-space (serine, glutamine and proline) di- and tri-residue repeats at the carboxy end of the protein. Searches of protein and DNA databases with the C-terminal end of NORF1 containing the SQ and SQP repeats did not reveal any significant matches. I speculate that it is possible that a SQ-space-SQ repeat could produce a highly hydrophillic region of polypeptide responsible for interactions with proteins that modifiy the activity of NORF1, that is, NORF1 might gain a function different from that of Upf1p. Alternatively, it might interact with a mammalian homologue of Upf2p.

Expression of NORF1

Northern blot analysis with the 3.6-kb NORF1 probe revealed a 5.5-kb and 3.6-kb transcript in all cell lines used in the analysis. Results of restriction and sequence analysis suggest that both transcripts encode NORF1. Although I did not quantitate the transcript levels in various cell lines, the result of the northern blot analysis in Figure 34 suggests that the ratio of the 3.6-kb and 5.5-kb transcripts might be different from cell line to cell line. It is possible that the 3.6-kb transcript represents a constitutively expressed mRNA, whereas levels of the 5.6-kb are regulated depending on the level of truncated polypeptides. It is tempting to speculate that sequences in the 3' UTR of the 5.6-kb species, about 2-kb, might be responsible for this regulation. The complete sequence of the 5' UTR of clone 5.6 and a repeat of northern blot analysis with different cell lines and tissues might support this idea. It is hard to imagine how a truncated protein can signal the upregulation of a mRNA. However, truncated and mis-folded polypeptides that bind to the ER chaperone protein BiP could trigger an intracellular signal pathway activating a protein that binds to the 5' UTR of the 5.6-kb NORF1 mRNA. Alternatively, NORF1 might directly be involved. We detected a putative tyrosine phosphorylation site in NORF1.

The finding that an antiserum against human NORF1 detects a 130-kDa polypeptide in human and mouse cells (Figure 41) suggests some protein conservation. This is supported by Southern blot analysis of mouse DNA with the human probe as well as by sequence data of the mouse NORF1 cDNA.

Interestingly, western blot analysis of rabbit proteins using the anti-human NORF1 peptide antiserum revealed a band of 115 kDa (data not shown). This may indicate that certain regions of the mouse and human NORF1 are dispensable for nonsense mRNA reduction. Therefore, the cloning and sequence of the rabbit NORF1 gene should identify regions that are different from mouse and human and possibly dispensable for NORF1 function.

Function of NORF1

To test whether NORF1 is able to promote nonsense mRNA reduction, I generated stable transfectants of a human NORF1 antisense cDNA into a mouse cell that expresses a nonsense μ mRNA. We found in one experiment and in one antisense transfectant that the expression of antisense-NORF1 mRNA correlated with a decrease of mouse NORF1 protein, an increase in nonsense μ mRNA, and the detection of a shorter polypeptide encoded by the nonsense μ mRNA. The reason why the level of nonsense μ mRNA was not restored to that of functional μ mRNA in FH could be that low levels of NORF1 protein can still be detected in antisense clone VXH.3. This raises the question of why the human *NORF1* antisense transcripts do not completely decrease the level of mouse NORF1 protein. One possibility is that differences in the nucleotide sequence between human and mouse *NORF1* result in a sense/antisense RNA hybrid that is only partially able to inhibit ribosomal translation of the mouse NORF1 protein. Indeed, partial sequence comparison of the mouse and human *NORF1* genes

reveals that the two cDNAs contain nucleotide differences. Nevertheless, the most straightforward interpretation of these functional tests is that *NORF1* is responsible for reducing nonsense μ mRNA levels. These results suggest that NORF1 is the first mammalian gene product that can affect the steady state level of a nonsense mRNA. However, this conclusion is based on the result obtained with this one VXH transfectant as well as from one western blot and northern blot of the indicated transfectants. The accuracy of the data obtained needs to be confirmed by repeating the western and northern blot analysis on these and possibly other independent transfectants.

Another approach involved transforming a yeast *upf1* mutant with a NORF1 expression plasmid to test for its ability to complement the *upf1* defect. The presence of the NORF1 expressing plasmid, and presumed NORF1 expression, did not result in yeast with an *upf1* phenotype as determined by no change in the Leu$^-$ phenotype. There may be a number of reasons that NORF1 did not complement the *upf1* defect. First, the yeast may not have expressed NORF1 mRNA and protein. This is unlikely as the yeast grew readily in the presence of galactose, indicating the presence of endogenous yeast factors needed for metabolism as well as induction of the NORF1 cDNA. Additionally, the NORF1 cDNA was found to be in the correct orientation in the expression vector (data not shown). Because experiments confirming the presence of NORF1 mRNA and protein were not performed, these possibilities cannot be excluded. Second, NORF1 may not complement *upf1* because it may not have

the ability to interact with factors also required to rapidly degrade nonsense mRNA. The alignment of NORF1 with *UPF1* (Figure 37) has revealed that not all regions of *UPF1* are conserved in NORF1. For example, both proteins have different N- and C-terminal tails. It could be that these are the regions of Upf1p that interact with the other *UPF* factors required to promote nonsense mRNA reduction. Because NORF1 lacks this region(s), it may not interact with other *UPF* factors.

In another attempt to determine if NORF1 promotes nonsense mRNA reduction, I transformed yeast containing a wild-type *UPF1* gene with a NORF1-expression plasmid to determine if NORF1 could act in a dominant-negative fashion on Upf1p. The presence of the NORF1-expressing plasmid, and presumed expression of NORF1 protein, did not result in yeast with an *upf1* phenotype. There may be a number of reasons that NORF1 did not act in a dominant-negative fashion. First, the yeast may not have expressed NORF1 mRNA and protein as discussed above. Second, there may be differences between NORF1 and Upf1p that do not allow direct interaction. The differences between Upf1p and NORF1 at their N- and C-terminal ends may prevent such interactions.

The final test of NORF1 function should be perfomed in a cell line that lacks NORF1 expresion. The next experiments to be done are 1) isolating of a genomic fragment and disrupting the *NORF1* gene in a mouse cell line, 2) finishing the sequence of the mouse *NORF1* cDNA to identify conserved and

possible functional residues, 3) isolating clones that encode proteins interacting with NORF1 in a yeast two-hybrid system, and 4) generating antibodies against mouse NORF1 to localize the intracelluar localization of NORF1 by immuno electron microscopy.

Models to Explain How NORF1 Reduces Nonsense mRNA levels

In yeast, nonsense mRNA seems to be selectively degraded in the cytoplasm in an *UPF*-dependent manner (reviewed in Maquat, 1995). However, cytoplasmic levels of nonsense mRNA in mammalian cells seem to be reduced by both nuclear and cytoplasmic events (reviewed in Maquat, 1995). NORF1, in concert with other NORF factors, could be involved in both events. It is tempting to speculate that the two zinc-finger-like motifs of the NORF1 may facilitate the recognition of nonsense codons either in mature mRNA in the nucleus and/or the cytoplasm or in looped-out nuclear pre-mRNA, in which the exons have been aligned to form a continuous translational reading frame (Dietz and Kendzior, 1994). One way in which the zinc-finger motif of NORF1 could be involved in nonsense codon recognition is that it could interact with a dsRNA region formed by the association of ribosomal subunits. The formation of dsRNA between ribosomal subunits is supported by experiments that have shown that the RNA components of mammalian ribosomes become less accessible to chemical modification after the 40S and 60S subunits have assembled into the 80S ribosome (Holmberg et al., 1994). From these results the authors believed that the RNA regions that are not modified are involved in rRNA subunit interactions.

Alternatively, rRNA could pair with tRNA molecules and form ds-rRNA/tRNA regions. That rRNA interacts with tRNA is suported by studies in prokaryotes that have shown that rRNA molecules are involved in tRNA selection and translational accuracy (reviewed by Noller, 1994). It may be possible that the zinc-finger motif of NORF1 binds to dsRNA or ds-rRNA/tRNA complexes. When these double-stranded regions are changed, either because tRNA is absent from the ribosomal A site, or ribosomal subunits disassociate, the binding of the zinc-finger to its double-stranded RNA binding site might be disrupted, which could result in the activation of the RNA helicase domain. Consequently, the helicase is activated, which leads to the degradation of nonsense mRNA as described below. That a zinc-finger motif could bind to dsRNA has been proposed for the ribosomal elongation factor eIF-2β. In eIF-2β, the zinc-finger motif is required for the recognition of the AUG start codon during the ribosome-mediated translational scanning process (Donahue et al., 1988). It has been suggested that the zinc-finger motif of eIF-2β could participate in start site selection by either scanning for an AUG or by stabilizing the AUG codon-anticodon interaction (a dsRNA interaction). Because a stop codon has no tRNA with an anticodon, NORF1 may only participate in interactions with ribosomal rRNA to recognize stop codons, similar to the way eIF-2β could interact with tRNA to stabilize AUG codon-anticodon interactions, and when activated by ribosomal disassociation, promote nonsense mRNA reduction (Figure 49). The importance

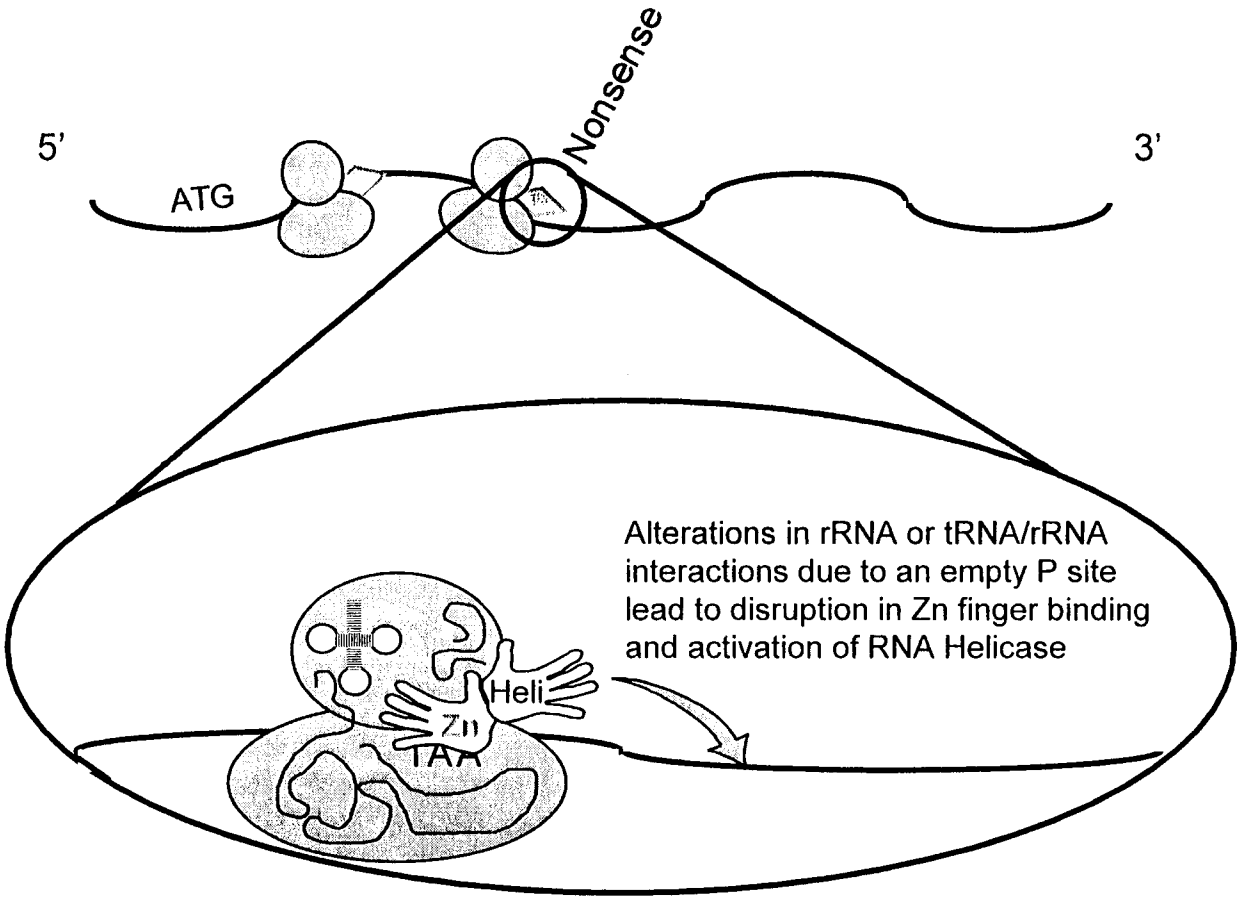


Figure 49. Model for activation of NORF1 leading to decay of an mRNA with a nonsense codon. The tRNA and rRNA present in the 80S ribosome are shown as squiggles.

of the zinc-finger motif in Upf1p has already been shown, because mutations in the zinc-finger motif of Upf1p eliminate its ability to recognize a nonsense codon but does not influence the RNA helciase activity of Upf1p (personal communication with K. Czaplinski) (see Table 2). These experiments were discussed in Chapter II.

The RNA helicase region of the Upf1p is critical for its ability to promote the decay of nonsense mRNAs (Leeds et al., 1992). It has been suggested that upon activation (discussed above), Upf1p could melt any secondary structure downstream of a nonsense codon by using its RNA helicase region, thereby leaving the mRNA exposed to nucleases (Figure 50.B; Peltz et al., 1993). For example, the ATP-dependent RNA helicase eIF-4A can render RNA susceptible to nucleases (Ray et al., 1985). NORF1 may also act in this way (Figure 50.B).

Another way in which Upf1p is thought to influence the degradation of a nonsense mRNA is to promote the rapid de-capping of the nonsense message (Muhlrad and Parker, 1994). One way to explain how the RNA helicase activity of NORF1 could facilitate the rapid de-capping of a nonsense mRNA is that Upf1p could unwind a *trans*-RNA interaction between the RNA portion of a RNA/protein complex and the capped end of a nonsense mRNA (Figure 50.C). This complex could somehow be involved in protecting the cap complex. Alternatively, the RNA helicase activity of NORF1 could unwind rRNA interactions in the ribosome which might result in the activation of other NORF factors that induce decapping (Figure 50.C). Yet, another way by which the RNA helicase activity of NORF1 could activate de-capping is to disrupt interactions

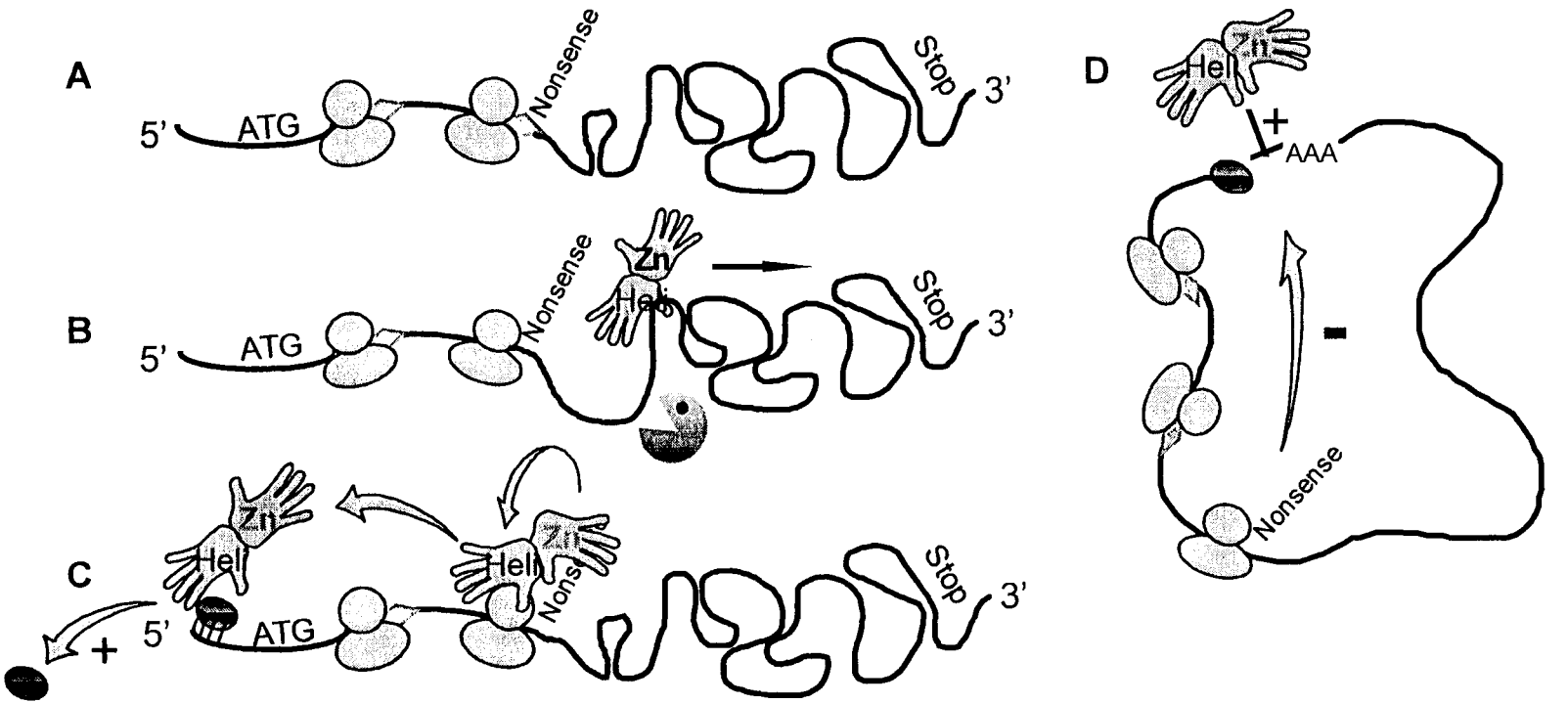


Figure 50. Models to explain NORF1 helicase action induces the rapid decay of a nonsense mRNA. The 5' and 3' end of a mRNA are indicated. The inactive NORF1 factor (indicated by a diamond) is associated with the translating ribosomes, becomes active (indicated by hands) after encountering a nonsense codon. **(A)** Schematic diagram of a nonsense mRNA with extensive secondary structure downstream of the nonsense codon. **(B)** Activation of NORF1 leads to removal of secondary structure downstream of the nonsense codon by the RNA helicase region, resulting in the production of a target for an endonuclease. **(C)** Activation of NORF1 induces RNA helicase activity. This results in the removal of a cap-protecting ribonucleo-complex, which results in decapping and degradation of the mRNA. Alternatively, NORF1 actication can lead to ribosomal disassembly and activation of other NORF factors. **(D)** Activation of NORF1 prevents 5' 3' RNA interaction, either directly (as shown) or indirectly by disruption of ribosomes. The inhibition of 5' 3' interactions leads to decapping and subsquent mRNA degradation.

between the 5' cap and 3' poly(A)-tail (Figure 50.D). This would expose the cap to a decapping enzyme and, subsequently, to 5'-3' exonuclease attack. That poly(A)/cap interactions might be required to protect mRNA from rapid degradation, comes from experiments that show that translation and deadenylation are prerequisites to decapping (Muhlrad et al., 1994) and from electron micrograph studies that show circular polysomes (Christensen et al., 1987).

If NORF1 can activate nonsense codon mRNA reduction, it raises the question of why mRNA with normal stop codons are not affected by the action of NORF1. It may be that NORF1 is a labile factor and may only be associated with an elongating ribosome for a limited time. The further a ribosome elongates, the greater its chance of loosing NORF1. After a certain distance, any ribosome encountering a stop codon will not recognize it as a nonsense codon because it lacks NORF1.

As mentioned above, nonsense mRNA in yeast seems to be selectively degraded in the cytoplasm in an *UPF1*-dependent manner (Peltz et al., 1994). However, cytoplasmic levels of nonsense mRNA in mammalian cells are apparently reduced by both nuclear and cytoplasmic events (reviewed in Maquat, 1995). If NORF1 is not found in the nucleus, other factors might be responsible for reducing nuclear levels of nonsense mRNA either by degrading mature nuclear mRNA or influencing the splicing of nonsense pre-mRNA (reviewed in Maquat, 1995). A mammalian homologue of Upf3p might be a

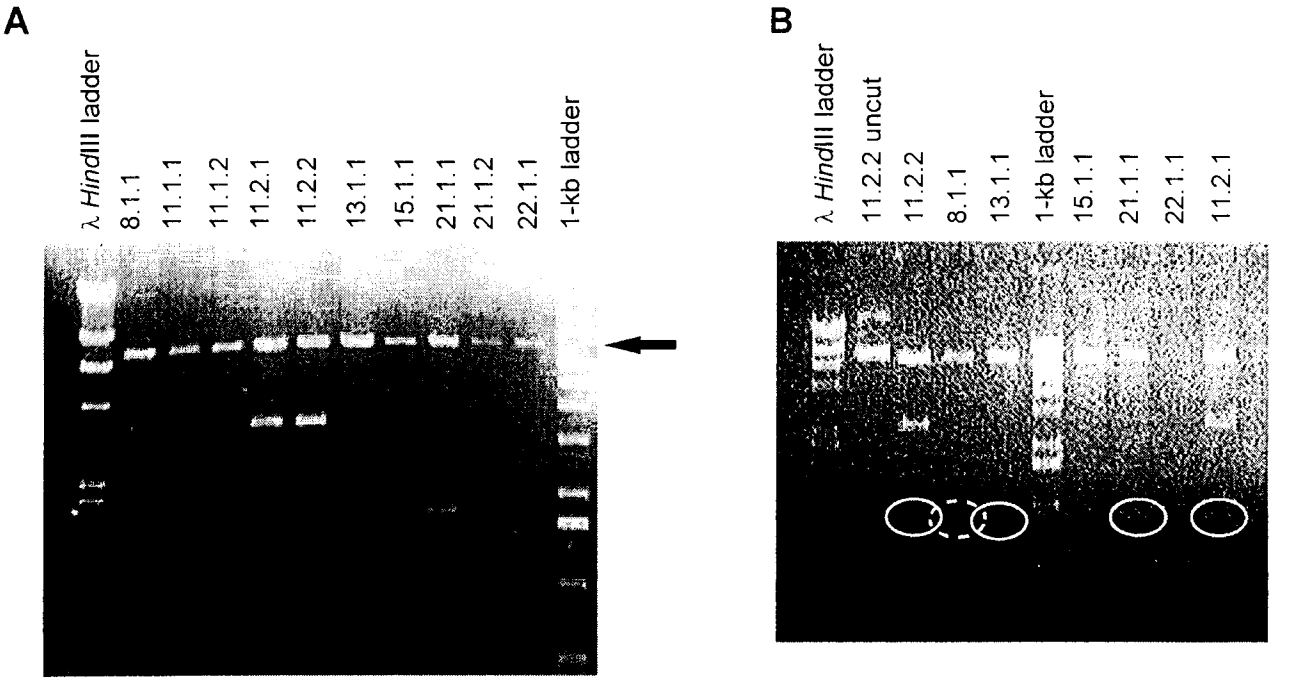
candidtate for a second factor. Upf3p contains a bi-partite nuclear localization sequence and thus, is thought to be present in the yeast nucleus (Lee and Culbertson, 1995).

NORF1: The Big Picture

The conservation of a protein that reduces levels of nonsense mRNA in lower (*S. cerevesiae and C. elegans*) and higher eukaryotes, such as mouse and human, implies an important biological function, presumably because it reduces nonsense mRNA levels and, thus, high levels of truncated polypeptides. Most truncated proteins might not be deleterious for a single cell. However, it might prevent in a dominant-negative fashion the assembly of signal-transducing receptors and prevent a cell from proceeding along a differentiation pathway or to fulfill its major physiologic function. If applied to the immune system, it is possible that nonsense immunoglobulin or T-cell receptor genes generated by DNA rearrangements could produce nonsense mRNA that can be translated into a truncated polypeptide. Such truncated Ig or TCR chains could interfere with the assembly of an Ig or TCR molecule, respectively, thereby eliminating the ability of the cell to respond to foreign antigens. The presence of a truncated polypeptide encoded by a stable nonsense mRNA correlates with a number of human diseases. For example, certain cases of Larons syndrome (Hashimoto et al., 1995), β-thalassemia (Hall and Thein, 1994), cancer associated with altered p53 expression (Kawasaki et al., 1994), Marfan syndrome (Dietz et al., 1994), and many others diseases are all thought to be the result of a dominant-negative protein product being translated from a mRNA containing a nonsense codon (see Table 1 for additional examples and references). It may be possible that these patients also have a defect in NORF1 (or another NORF gene) making

them susceptible to protein products produced from nonsense mRNAs that are not eliminated. Therefore, NORF1 not only protects a cell from nonsense mRNAs but also from the deleterious effect of truncated polypeptides encoded by nonsense mRNA.

APPENDIX

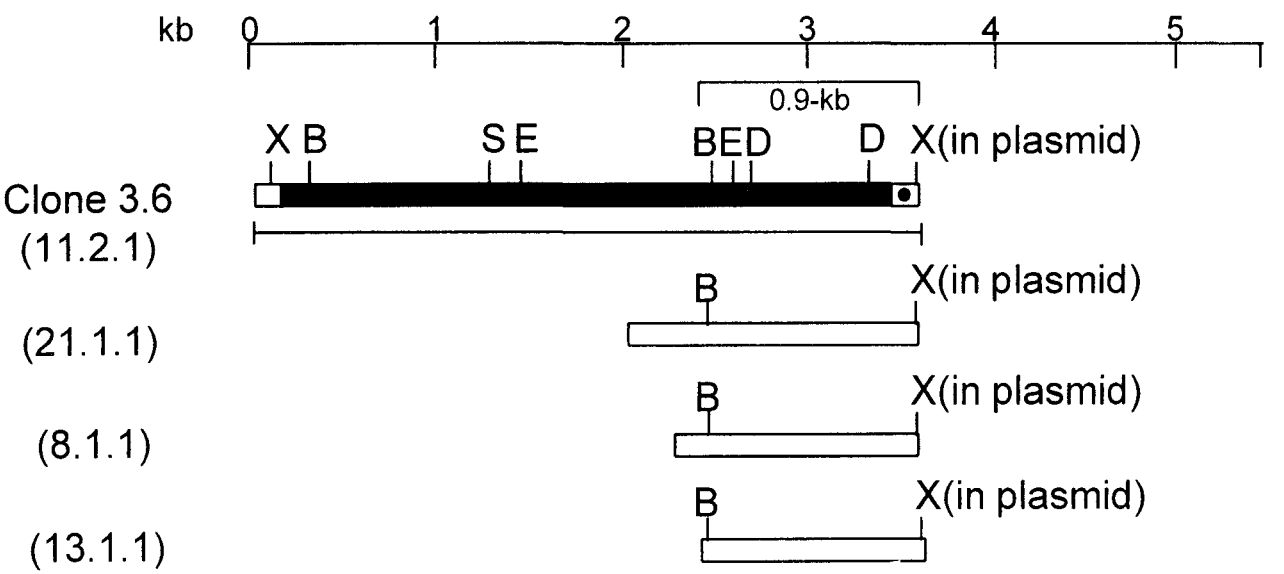


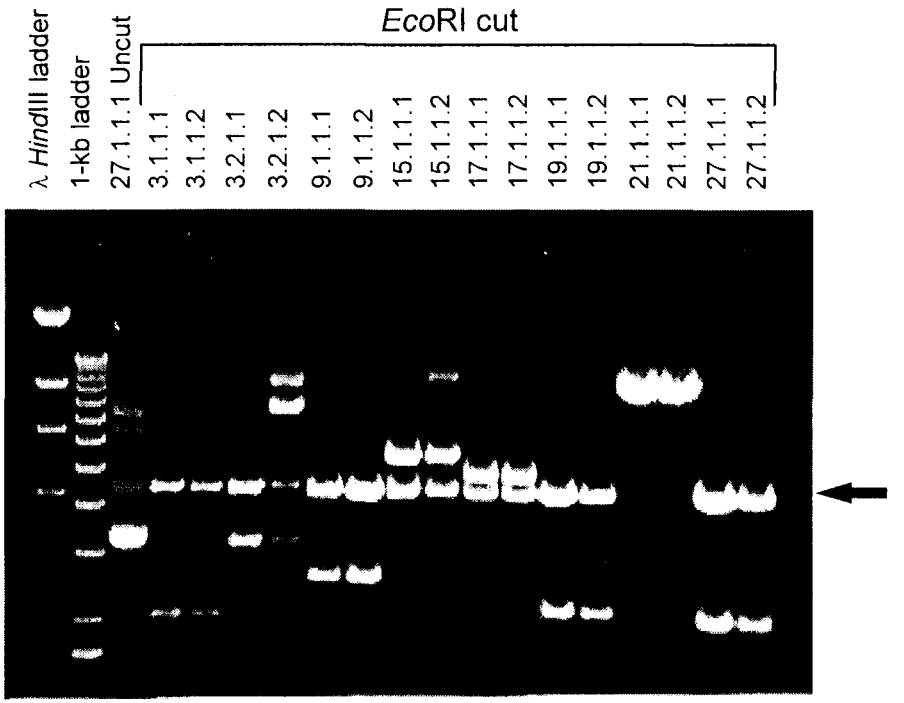
Additional Figure 1. Determination of insert size and restriction enzyme mapping of λYES cDNA clones that hybridize with EST clone R13609. **(A)** Determination of insert size in phage excised plasmid DNA. Plasmid DNA was analyzed by TAE/agarose gel electrophoresis using *Xho*I digestion. Molecular weight markers are in the indicated lanes. The band representing the λYES vector DNA is indicated by the arrow on the right. **(B)** Mapping of inserts in phage excised plasmid DNA by C. Kampershorer (5/16/96). Plasmid DNA was digested with *Bst*XI and *Xho*I and analyzed by TAE/agarose gel electrophoresis. The faint 900 bp fragment identified in certian lanes is indicated by a white circle. **(C)** Table listing the phage clone picks and their insert sizes in kb. **(D)** Schematic representation of results from restriction enzyme mapping in **B** using diagnostic *Bst*XI (B) and *Xho*I (X) mapping. Clone 22.1.1 gave no results and clone 11.1.1 was not tested (CK).

C

Clone	Insert size in kb
11.2	3.6
22.1	1.9
21.1	1.8
11.1	1.7
8.1	1.3
13.1	0.8
15.1	?

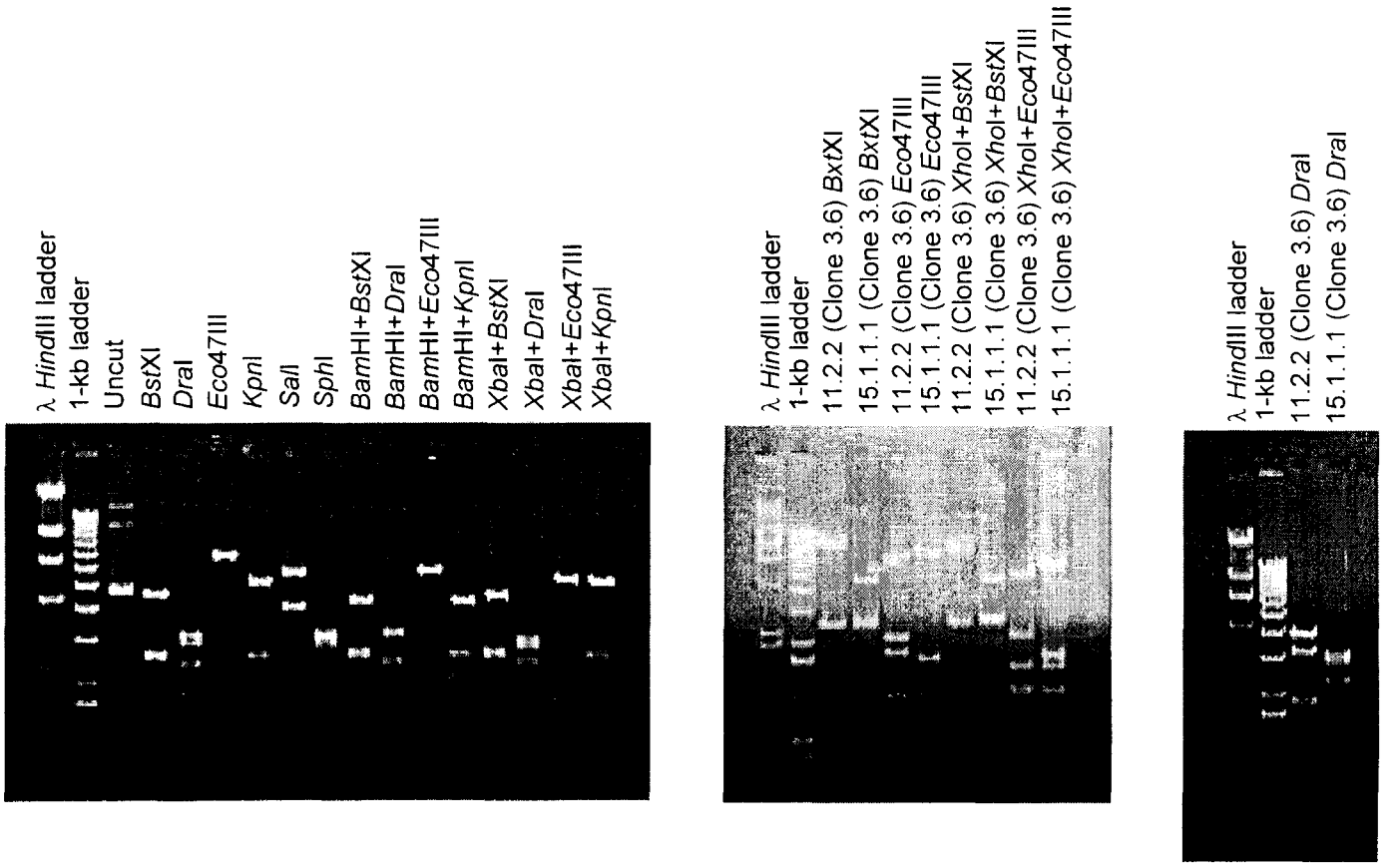
D



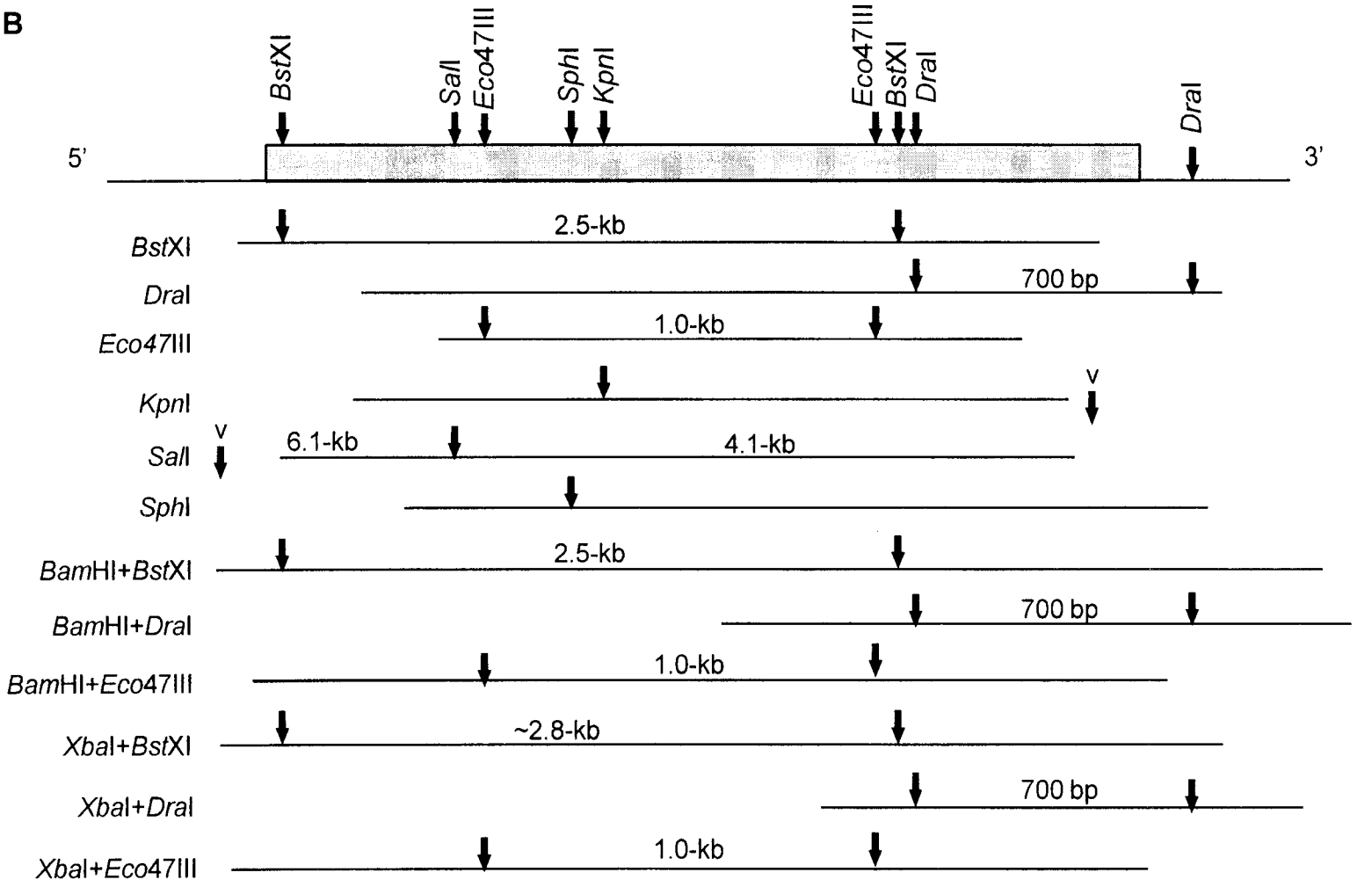


Additional Figure 2. DNA restriction enzyme analysis of the set of human NORF1 clones isolated from the Jurkat random-primed cDNA library. Lanes and DNA analyzed are indicated using TAE/agarose gel electrophoresis. Molecular weight markers are in the indicated lanes. The band representing the 4.6-kb pBluescript SK vector is indicated on the right by the arrow.

A



Additional Figure 3. DNA restriction enzyme analysis of human 5.5-kb NORF1 clone (15.1.1.1). **(A)** Lanes and DNA analyzed are indicated using TAE/agarose gel electrophoresis. Molecular weight markers are in the indicated lanes. **(B)** Schematic diagram of DNA fragments identified within human 5.5-kb NORF1 clone. Restriction enzymes used are indicated and (v) indicates the presence of the restriction enzyme cut site within the vector (λYES).


B
BstXI
SalI
Eco47III
SphI
KpnI
Eco47III
BstXI
DraI
DraI
5'
3'
BstXI
2.5-kb
DraI
700 bp
Eco47III
1.0-kb
KpnI
V
SalI
V
6.1-kb
4.1-kb
SphI
BamHI+BstXI
2.5-kb
BamHI+DraI
700 bp
BamHI+Eco47III
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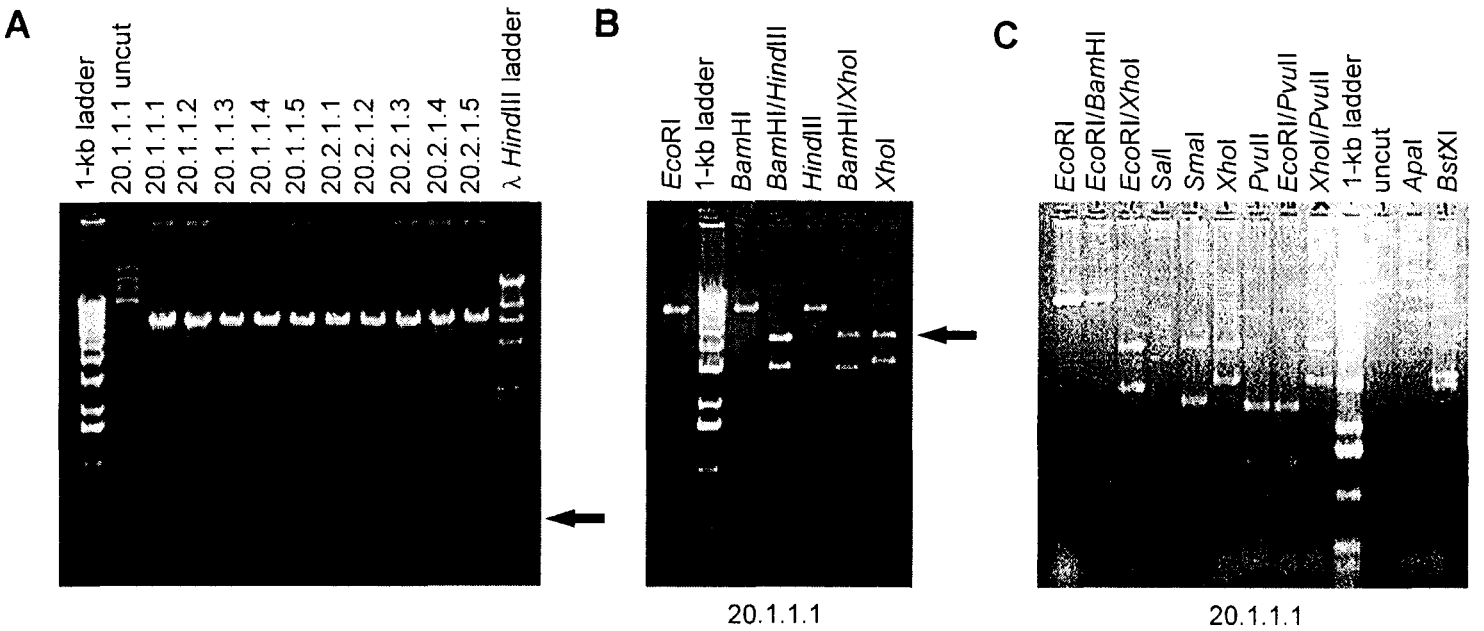
XbaI+DraI
700 bp
XbaI+Eco47III
1.0-kb



Additional Figure 4. DNA restriction enzyme analysis of mouse 4.5-kb NORF1 clone (20.1.1.1). **(A)** Initial analysis of insert size in mouse cDNA clones 20.X.X.X. Plasmids were digested with *Eco*RI to excise the insert but as the results show, apparently only linearized it. Small non-photographable band at ~450 bp is indicated by the arrow on the right. Molecular weight markers are shown in the indicated lanes. **(B, C, D, E, and F)** DNA restriction enzyme analysis of mouse NORF1 clone 20.1.1.1. Mouse NORF1 was digested with the indicated enzymes and mapped. (B) 4.5-kb cDNA insert is indicated by the arrow on the right. Molecular weight markers are shown in the indicated lanes. **(G)** Schematic representation of the pBluescript SK plasmid containing the 4.5-kb NORF1 cDNA clone.

D

λ *Hind*III ladder
*Bgl*II
*Bst*XI
*Dra*I
*Eco*47III
*Pst*I
*Sal*I
*Sma*I
*Xba*I
*Xho*I

20.1.1.1

E

λ *Hind*III ladder
1-kb ladder
uncut
*Bgl*II
*Bgl*II/*Bam*HI
*Bgl*II/*Hind*III

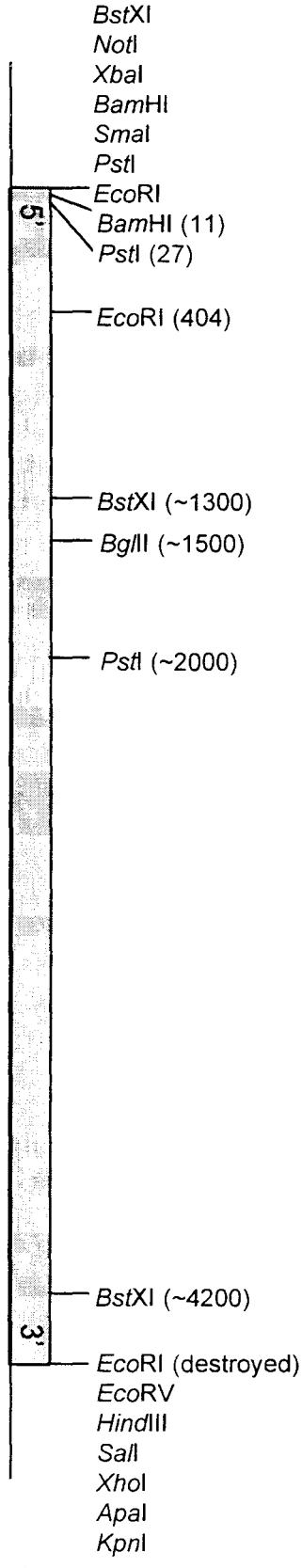
20.1.1.1

F

λ *Hind*III ladder
1-kb ladder
uncut
*Bam*HI
*Hind*III
*Bst*XI
*Bam*HI/*Hind*III
*Bam*HI/*Bst*XI
*Hind*III/*Bst*XI

20.1.1.1

G


BstXI
NotI
XbaI
BamHI
SmaI
PstI
EcoRI
BamHI (11)
PstI (27)
EcoRI (404)
BstXI (~1300)
BglII (~1500)
PstI (~2000)
BstXI (~4200)
EcoRI (destroyed)
EcoRV
HindIII
SalI
XhoI
ApaI
KpnI
5'
3'

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This dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

9/25/96
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