

ABSTRACT

Diane S. Bonner. MAPPING OF THE HUMAN MITOCHONDRIAL TRANSLATION INITIATION FACTOR 2 GENE (MTIF2) TO HUMAN CHROMOSOME 2. (Under the direction of Mary A. Farwell, Ph.D.) Department of Biology, East Carolina University, Greenville, NC January 1999.

Human Mitochondrial Translation Initiation Factor 2 (MTIF2) is a nuclear gene. Its product, IF2_{mt}, is needed for the process of translation in mitochondria. IF2 is made on cytoplasmic ribosomes in the cytosol, and then imported into the mitochondrial matrix. It catalyzes the binding of fMet-tRNA to the ribosomal small subunit. Complete cDNAs encoding human IF2_{mt} have been isolated from cDNA libraries, cloned and sequenced. (Ma and Spremulli, 1995). Several genomic clones containing MTIF2 have been isolated from a Lambda FixTM library and the DNA sequenced.

The chromosomal location of the gene was found using a somatic cell hybrid panel. Each DNA sample contained mouse chromosomes and one intact human chromosome. The polymerase chain reaction (PCR) technique, using two PCR primers designed from MTIF2 sequence data showed the location of the gene to be on chromosome 2. The PCR primers anneal to DNA sequences flanking the intron/exon junction near the 3'-end of the gene. Human parental DNA served as a positive control and mouse genomic DNA as a negative control.

The fluorescence *in situ* hybridization (FISH) technique confirmed the location. Four human genomic clones containing overlapping fragments were used as probes on human metaphase chromosome spreads. A more precise location of the gene was achieved by G-banding of the metaphase spreads used in FISH. MTIF2 was mapped to the short arm of chromosome 2 in the region 2p16 to 2p14 (Bonner et al., 1998).

**MAPPING OF THE HUMAN
MITOCHONDRIAL TRANSLATION
INITIATION FACTOR 2 GENE (MTIF2)
TO
HUMAN CHROMOSOME 2**

A Thesis

Presented to

**the Faculty of the Department of Biology
East Carolina University, Greenville, NC**

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Molecular Biology/Biotechnology

by

**Diane S. Bonner
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Factor 2 Gene (MTIF2) to Human Chromosome 2**

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Diane S. Bonner

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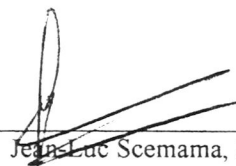
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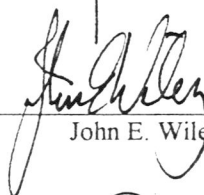
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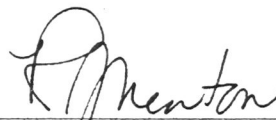
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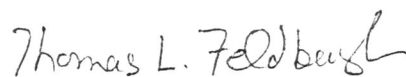
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INTRODUCTION

Mitochondria

The mitochondrion is an organelle found in nucleated cells in eukaryotes. Its principal function is oxidative phosphorylation, that is, converting energy from the oxidation of food into the synthesis of ATP, the energy source used to drive cellular reactions. It also contributes to the biosynthesis of many other metabolites used by the cell.

Mitochondria, though found in all tissues, are most abundant in skeletal muscle, liver, heart, kidney (Ma and Spemulli, 1995), and even sperm cells, wherever there is a high demand for energy. They occupy a large volume of the cell; for example, there are 1000 to 1600 mitochondria in a liver cell (DeRobertis and DeRobertis, 1987).

Mitochondria are maternally inherited because, at fertilization, the egg cell contributes the cytoplasm to the zygote. Mitochondria can be spherical or elongate (Ord and Smith, 1982; Flickinger, 1968), are remarkably mobile and plastic (Bereiter-Hahn, 1990), are constantly changing their shape and can fuse with one another and then separate again (Chida and Ueda, 1986; Malcom and Wetherbee, 1987). In some cells, such as cardiac muscle, they are fixed in one position in order to provide ATP directly to a site of unusually high ATP consumption. Mitochondria are bound by two highly specialized membranes that create two separate mitochondrial compartments. The outer membrane has large aqueous channels that are permeable to all molecules of 5000 daltons or less. The intermembrane space is chemically equivalent to the cytosol. The inner membrane is highly specialized and selectively permeable. It contains a variety of transport and other

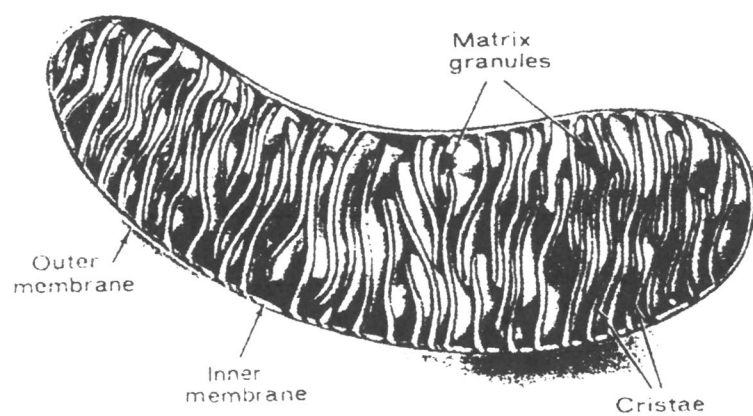
proteins, and is folded into numerous cristae, which greatly increases its total surface area (Figs. 1 and 2).

Mitochondria cannot be synthesized *de novo*, but replicate by fission (Osumi and Sando, 1969; Storrie and Attardi, 1973; Kuroiwa, 1982; Palade, 1983; Attardi, 1988). They have their own DNA, and it has been completely sequenced in man, mouse, rabbit, yeast, cow (Bibb et al., 1981; Anderson et al., 1981, 1982; Kozak, 1983; de Zamaroczy and Bernardi, 1985; Grivell, 1987) and others. Mammalian mitochondrial genomes are composed of approximately 16,500 bases, whereas plants have much larger mitochondrial genomes. In mammals, the genome codes for 13 proteins, 22 transfer RNAs and 2 ribosomal RNAs (Ojala et al., 1980; Borst and Grivell, 1981). Each mitochondrion has multiple copies of its DNA. However, because of its size, the total amount of mitochondrial DNA relative to nuclear DNA is less than one percent. Although most mitochondrial DNA in animals is circular, there are some that are linear, such as in *Hydra* (Warrior and Gall, 1985), and in ciliate protozoans, such as *Tetrahymena* (Suyama et al., 1985).

Although they have their own genetic system, mammalian mitochondria produce only a small proportion of their own proteins. The proteins encoded by the mitochondrial genome are synthesized in the mitochondrial matrix and become inserted into the inner mitochondrial membrane as part of integral membrane proteins. Thirteen mRNAs, rRNAs and tRNAs are synthesized in the mitochondrion, but the RNA polymerase, the ribosomal proteins and the tRNA synthetases are nuclear encoded and translated in the cytoplasm (Attardi and Schatz, 1988). They are transported into the inner matrix in a

Fig. 1. Electron micrograph of a mitochondrion from the pancreas cell of a bat, showing the cristae, matrix, and matrix granules. $\times 79,000$ (Bloom and Fawcett, 1975).

Fig. 2. Drawing of a longitudinal section of a mitochondrion showing the smooth outer membrane and an inner membranes with slender folds called cristae that extend into the interior of the organelle (Bloom and Fawcett, 1975).



two-stage process at contact sites that join the inner and outer membranes (Schatz and Butow, 1983; Schleyer and Neupert, 1985; Hartl and Neupert, 1990; Pfanner et al., 1992). The proteins are imported in an unfolded state guided by chaperones. Once inside, other chaperones assist in their final folding (Ellis, 1987; Chirico et al., 1988; McMullin and Hallberg, 1988; Murakami et al., 1988; Ellis and Hemmingsen, 1989; Weinhues and Neupert, 1992; Stuart, 1994). Assembly of polypeptides made inside the organelle with those made in the cytoplasm requires a great deal of coordination between two distinct compartments. How the mitochondrion and the nucleus communicate is still not known. In addition, the origin of the mitochondrion is still debated (Attardi, 1985; Gray, 1989; Payton and McEwen, 1996).

According to the serial endosymbiosis theory (SET), the ancestral eukaryotic cell type was believed to be an anaerobic, prokaryotic cell capable of ingesting solids through its mobile cell-surface activities (Margulis, 1970). Such a cell is postulated to have engulfed respiring prokaryotic (purple nonsulfur bacteria) cells, leading to a mutually beneficial, or symbiotic, relationship between the host and its endosymbionts within the cell. The host provided nutrients and protection, and the endosymbionts provided the energy efficient aerobic respiratory pathway to the anaerobic host in a world undergoing an atmospheric change from anaerobic to aerobic conditions. Evidence to support this hypothesis is based on strong sequence similarities in cytochrome c, ferredoxin and 5S rRNA between mitochondria and purple bacteria.

The genetic machinery of mitochondria closely resembles that of prokaryotic cells; however, since the majority of the proteins functioning in energy synthesis in the

mitochondria are encoded by the nuclear genome, it is possible that during evolution the nuclear genome has acquired genes from the mitochondrion. Therefore the genes for all the proteins made in the mitochondria may eventually move to the nucleus. There is evidence that gene movement is occurring, since mammals, arguably the most evolutionarily advanced species, have the smallest mitochondrial genomes.

Mitochondrial genome size varies over more than a 150-fold extent. Nematode has a low of 14.3 kbp (Wolstenholme et al., 1987), with human, mouse, bovine and *Xenopus laevis* being similar in size at 16.5-17.6 kbp (Gray, 1989). Yeast has approximately 80 kbp (Cantatore and Saccone, 1987), and it ranges to an estimated high of 2400 kbp in cucurbit (Ward et al., 1981). Plants have the largest and the most complex mitochondrial genomes (Pring and Lonsdale, 1985; Newton, 1988).

Translation

Protein biosynthesis occurs by the process of translation. Translation converts the language of genetic information embodied in the base sequence of a messenger RNA molecule into the amino acid sequence of a polypeptide chain. Proteins consist of one or more polypeptide chains. Many function as highly specific biological catalysts called enzymes, which control cellular metabolism. Others are nonenzymatic such as hemoglobin, collagen, hormones and immunoglobulins, and serve structural and other roles. Proteins are synthesized on ribosomes. Ribosomes and ribosomal subunits are complex structures composed of RNAs and proteins (Table 1). Many of the proteins and the rRNAs are concerned with establishing the overall structure to bring the various

Table 1. Sizes and protein compositions of ribosomes from various sources.

Source	Sedimentation coefficients			No. of proteins	
	Small subunit	Large subunit	Complete ribosome	Small subunit	Large subunit
<i>E. coli</i>	30S	50S	70S	21	32
Chloroplasts of					
higher plants	30S	50S	70S	22-24	32-35
Mitochondria					
Yeasts	37S	50S	70-74S	33	38
Plants	40S	60S	78-80S		
Mammals	~30S	~40S	55-60S	33	52
Eucaryotic cytoplasm					
Yeasts	40S	60S	80S	30-32	41-44
Rodents	40S	60S	80S	~31	~49

(Kozak, 1983)

active sites into the correct orientation and do not necessarily participate directly in the synthesis. The ribosome possesses several active centers; each constructed from a particular group of proteins associated with a region of ribosomal RNA. Ribosome conformation is flexible during protein synthesis (Noller, 1984, 1991; Hershey, 1991; Merrick, 1992). Each ribosome is made up of two unequal subunits (Fig. 3). The ribosome sizes are reported in Svedberg units (See Appendix A). A 50S and a 30S subunit comprise the prokaryotic ribosome, which has a value of 70S. The eukaryotic ribosome is 80S and is composed of a 60S and a 40S subunit. In mitochondria these subunits consist of a 28S and a 39 S (O'Brien and Matthews, 1976).

The process of translation is characterized by three distinct phases: initiation, elongation, and termination (Fig. 4). At each stage, the energy driving the assembly process is provided by GTP hydrolysis (Hershey, 1991). Initiation involves the reactions that precede formation of the peptide bond between the first two amino acids of the protein. This is the slowest step in protein synthesis and usually determines the rate at which mRNA is translated. The elongation step includes all the reactions from synthesis of the first peptide bond to addition of the last amino acid. The addition of an amino acid is the most rapid state in protein synthesis. Termination encompasses the steps that are needed to release the completed polypeptide chain.

Initiation of translation involves a mRNA molecule, a specific charged initiator transfer RNA, a GTP molecule, initiation factors (IFs), and the ribosomal subunits. The methionyl-tRNA used to start initiation is formylated in the prokaryotic and

Fig. 3. Ribosomal subunits and their proteins.

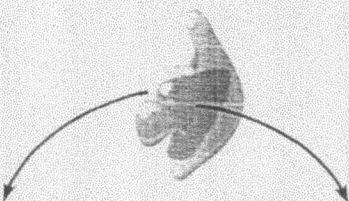
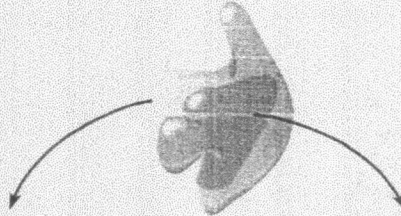
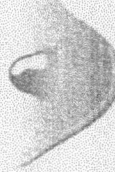
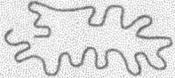
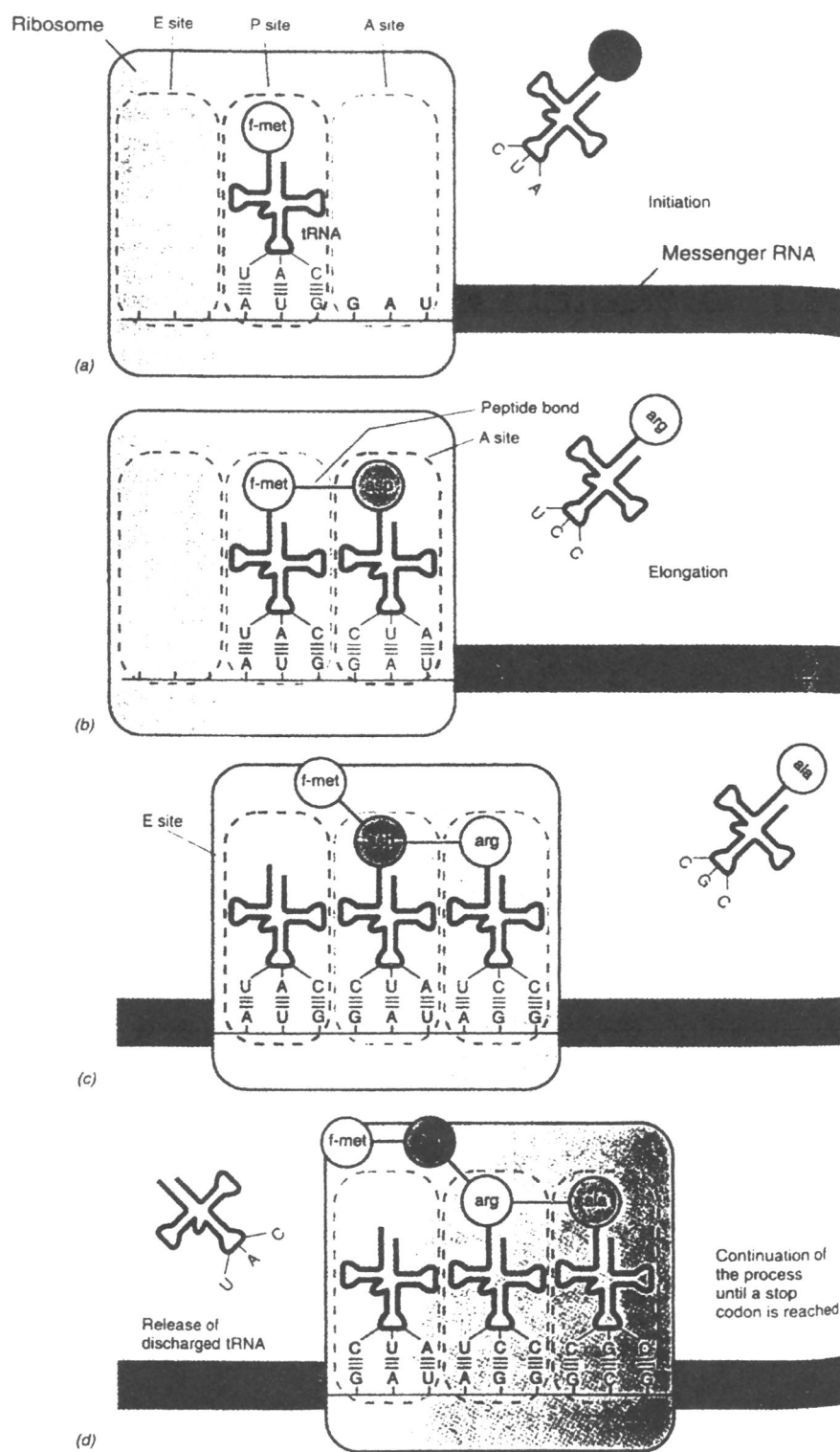
PROKARYOTES		EUKARYOTES	
70S (2.5×10^6 MW) 		80S (4.0×10^6 MW) 	
50S 1.6×10^6 MW 	30S 0.9×10^6 MW 	60S 2.7×10^6 MW 	40S 1.3×10^6 MW 
23S rRNA (~2900 nucleotides)  + 31 proteins  + 5S rRNA (120 nucleotides) 	16S rRNA (~1540 nucleotides)  + 21 proteins 	28S rRNA (~4800 nucleotides)  + 50 proteins  5S rRNA (120 nucleotides) + 5.8S rRNA (160 nucleotides)  	18S rRNA (~1900 nucleotides)  + 33 proteins 

Fig. 4. Pictorial representation of the three steps involved in translation: initiation, elongation, and termination (Brock et al., 1994).



mitochondrial systems. The methionine has a formyl group attached to its amino group, and the blocked amino acid then prevents the initiator from participating in chain elongation. The initiator tRNA has other unique features: the last two bases in the stem to which the amino acid is connected are not base-paired and there is a series of three GC pairs in the stem preceding the anticodon loop which are highly conserved (Sprinzl et al., 1987). If these base pairs are changed, it leads to a different loop conformation (Seong and RajBhandary, 1987). In the initiation complex, the small subunit binds the mRNA in such a way that the initiation codon on the mRNA lies within the part of the P site of the subunit. The only charged tRNA that can become part of the initiation complex is the initiator tRNA. A different methionyl-tRNA recognizes the AUG codons in internal locations during elongation.

The initiation factors used in translation are not part of the ribosome, but are required to enhance the binding affinity of the various translational components. Most ribosomes, when not actively involved in translation, are dissociated into their large and small subunits. The small ribosomal subunit binds to several initiation proteins, and this complex in turn binds to mRNA. Each ribosome has two sites for association with transfer RNA molecules. The end of the tRNA which carries the amino acid is located in the large subunit while the anticodon at the other end interacts with the mRNA bound by the small subunit. There are three other sites, the aminoacyl (A) site, the peptidyl (P) site, and the exit (E) site. Each has different roles in translation (Fig. 5). The A-site is generally the one into which aminoacyl tRNAs enter the ribosome-mRNA complex and is specified by the second mRNA codon. The P-site is the one from which the tRNA

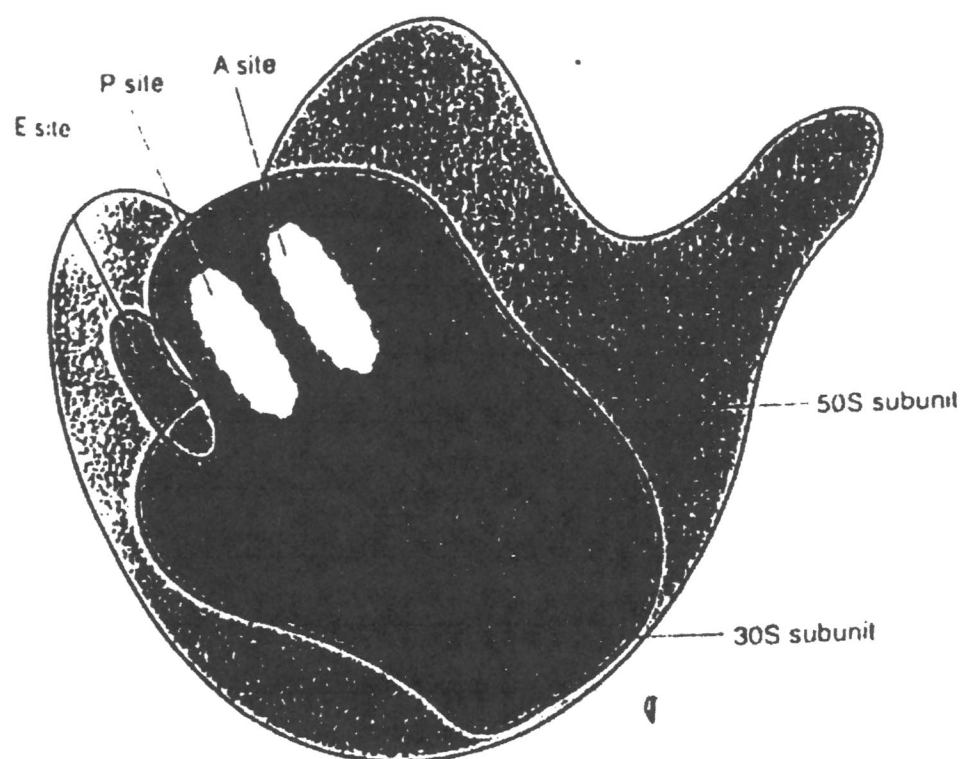
donates an amino acid to the growing chain. The initiator tRNA binds initially in the P-site of the ribosome. A third site, the E (exit) site is thought to be the final location where the deacylated tRNA molecule rests before exiting the ribosome for recycling.

Prokaryotic translation

Translation has been studied in eukaryotes and prokaryotes and there are many differences between the two organisms. Little is known about the mitochondrial system but it appears that translation in mitochondria better resembles the prokaryotic system. In *Escherichia coli*, the model for organellar translation, 30S and 50S subunits join to make a 70S ribosome. The 50S subunit is composed of a 23S and a 5S subunit and 31 proteins. The 30S subunit is composed of a 16S rRNA and 21 proteins. A feature of the primary structure of rRNA is the presence of methylated residues, about 10 of them in the 16S subunit, and 20 in the 23S subunit. These residues are located mostly toward the 3' end, and are in close proximity when the ribosome is assembled.

Three initiation factor proteins (IF1, IF2, and IF3) bind contiguously near the 3' end of the 16S rRNA at adjacent or overlapping sites that are located at the interface of the two subunits. Following binding of the factors, the charged formylmethionyl initiator tRNA (fMet-tRNA) and mRNA specifically bind to the factor-ribosome complex (Czernilofsky et al., 1975; Langberg et al., 1977; Maitra et al., 1982). The binding of the mRNA to the ribosome involves a special sequence, called the Shine-Dalgarno sequence (Shine and Dalgarno, 1974; Steitz and Jakes, 1975), on the mRNA. It consists of up to six ribonucleotides and precedes the initial start codon (usually AUG, but it can be GUG

Fig. 5. Structure of the ribosome, showing the A, P, and E sites on the large subunit of the ribosome (Brock et al., 1994).



or UUG). The distance between this sequence and the start codon is significant; it must be within ten bases upstream of the start codon. This sequence, which is purine rich, base pairs to the 3' region of the 16S ribosomal subunit to form a binary complex. In fact, an intact and unaltered 3' end of the 16S RNA subunit is indispensable for protein synthesis. This binding helps diminish the space to be searched by the 30S subunit for the initiation codon on the mRNA.

IF2 facilitates the binding of initiator-tRNA to the 30S subunit in response to the AUG triplet. IF2 is the largest of the three initiation factors and is probably associated with the P site (Grunberg-Manago, 1980; Gualerzi and Pon, 1981). Because the 30S subunit displays a high affinity for IF2, IF2 is thought to act while bound to the 30S subunit rather than as a free molecule (Guillon, 1996). IF2 also has a binding site for the GTP molecule. The three molecules (IF2, GTP, tRNA) in a 1:1:1 ratio with one molecule of 30S and the mRNA form the preinitiation complex (Fakunding and Hershey, 1973). After IF2 binds the tRNA, IF3 is released. The 50S subunit then joins and forms the 70S initiation complex. The presence of GTP prevents tRNA from participating in peptide bond formation. When the GTP molecule is hydrolyzed to GDP, the GDP-IF2 complex is released, altering the configuration of the complex and making the tRNA active. IF2, by kinetic effects, discriminates among charged, uncharged, and elongator tRNAs. IF1 helps in the release of the IF2 factor (Maitra et al., 1982). Due to the concomitant ejection of IF1 and IF3, formation of the 70S-initiation complex is virtually irreversible. With the charged formylated tRNA in the P site, the reading frame is set so that all

subsequent groups of three ribonucleotides are translated accurately (Gualerzi and Pon, 1990).

The other initiation factors are less well characterized than IF2. IF1 is thought to stabilize the binding of IF2 to the 30S complex. The IF3 factor, by concentration effects, discriminates between initiator and elongator tRNAs, and restricts elongator tRNAs from accessing the initial P site (Hartz et al., 1989). IF3 also helps in the formation of hydrogen bonds between the 16S subunit and the Shine-Dalgarno region of the mRNA (Szer et al., 1976; Steitz et al., 1977; Maitra et al., 1982). This bonding anchors the 30S subunit in a position leading directly to the positioning of the start codon in the ribosomal P site, where it interacts with the initiator tRNA. When the initiator tRNA is bound, the mRNA becomes firmly fixed in the downstream region, particularly at the A-site codon. The mRNA has been proposed to run along the head-body junction of the 30S subunit with the A- and P-site anticodons located roughly in the middle facing the 50S subunit (McCarthy and Brimacombe, 1994).

IF2

E.coli IF2 is a monomeric protein that has been shown to occur in three forms (α , β , γ) in the cell, with molecular weights of about 100,000, 80,000 and 70,000, respectively (Plumbridge et al., 1985). All three forms are transcribed and translated from the same gene, *infB*. Both the α and β forms are identical in DNA sequence except for the N-terminus which is missing from the β form (See Appendix B).

Eukaryotic translation

The mechanism of initiation complex formation in eukaryotes is quite different from the process observed in prokaryotes. The main differences in the eukaryote are: 1) the ribosomes are larger; 2) more protein initiation factors are involved; 3) the methionyl initiator tRNA is not formylated; 4) there is a 7-methyl guanosine triphosphate “cap” structure at the 5' end of the mRNA; and 5) the mRNAs are monocistronic in nature (Shatkin, 1976; Maitra et al., 1982). In the eukaryotic cytoplasmic system, the initial interaction of the mRNA with the 40S subunit (18S rRNA and 33 proteins) is mediated by at least eight initiation factors (Kozak, 1983). One of the factors, eIF4G, binds to the cap structure of the message and mediates interaction between the 5' end of the capped mRNA and the 3' end of 18S RNA (Nakashima et al., 1980). There is abundant evidence that the cap moiety enhances the efficiency of initiation by increasing the affinity of the mRNA for the ribosomal subunits (Muthukrishnan et al., 1978; Maitra et al., 1982). It has been postulated that the evolution of the cap structures in higher cells may facilitate ribosome binding of mRNA and replace the mRNA: rRNA interaction that occurs in prokaryotes. An associated factor moves along the 5' end of the message removing any double stranded regions that would interfere with the movement of the ribosome as it scans for the initiation codon (Ilan and Ilan, 1976; Maitra et al., 1982).

Initiation of protein synthesis in eukaryotes involves the formation of a trimeric complex between ribosomes, mRNA, and methionyl-tRNA. The binding of methionyl-tRNA to 40S ribosomes is promoted by initiation factor eIF2, which first forms a ternary complex with GTP and Met-tRNA. The ternary complex binds to a 40S ribosomal

subunit, followed by mRNA binding to form a 43S preinitiation complex. The 40S ribosome evaluates AUG codons sequentially. The position of an AUG codon relative to its 5' end of the mRNA is as important as its context (Kozak, 1991). The 60S ribosomal subunit, which is composed of the 28S, 5S and 5.8S subunits and about 50 proteins, joins the 43S preinitiation complex to form the 80S initiation complex. It is preceded by hydrolysis of the GTP molecule, which is bound to the eIF2 factor. eIF5 promotes GTPase reaction and the eIF2-GDP binary complex is then released (Raychaudhuri and Maitra, 1986; Gaspar et al., 1994). In order for eIF2 to recycle and catalyze another round of initiation, the GDP bound to eIF2 must exchange with GTP by way of a reaction catalyzed by eIF2B. Since Met-tRNA binding is a common step in the translation of all mRNAs, eIF2 functions as an important regulatory protein for controlling overall protein synthesis rates. If eIF2 is phosphorylated, it is not able to make ternary complexes or transfer Met-tRNA to 40S initiation complexes (Ohtsuki et al., 1980; Maitra et al., 1982).

eIF2

Mammalian eIF2 exists primarily as a 145,000 trimeric protein and is composed of three nonidentical subunits, α , β , and γ . In eukaryotic translation, the eIF2 α -subunit contains the site of phosphorylation, which can be used for translational regulation. The β -subunit's function is not known. The γ -subunit is the site for GTP and Met-tRNA binding (See Appendix C). It is homologous to EFTu in *E.coli*.

Both *E.coli* IF2 and eukaryotic cytoplasmic eIF2 are members of the class of proteins referred to as G-proteins, which bind to and hydrolyze GTP in their G-domain.

The G-domain of most proteins is situated in the N-terminal part of the molecule, but in prokaryotic proteins, the G-domain is localized in the central part (Bourne et al., 1990). The requirement of mitochondrial IF2 for GTP and the inhibition of its activity by GDP suggest that the organellar factor falls into this class of proteins. In the prokaryotic system, subunit joining occurs prior to GTP hydrolysis and the release of IF2 occurs following 70S initiation complex formation. In contrast, in the eukaryotic cytoplasmic system, eIF5 promotes the hydrolysis of the GTP present in the ternary complex on the 40S subunit. This interaction then allows the release of eIF2 prior to the joining of the 60S subunit. In the mitochondrial system the subunit-joining step does not require GTP hydrolysis and, in this respect, the mitochondrial system resembles that of prokaryotes more closely than that found in the eukaryotic cytoplasm (Liao and Spremulli, 1991).

Mitochondrial translation

In contrast to the other systems, not much is known about protein synthesis in the mitochondria. The process resembles the bacterial system in the use of the formylated initiator tRNA; however, this system has a number of unique features that distinguish it from other translational systems. Mammalian mitochondrial DNA has a single gene coding for tRNA^{Met} species, and this gene is thought to give rise, in an unknown manner, to the synthesis of both initiator and elongator tRNA^{Met} species. This unusual feature has not been found in any other known protein synthesizing system including those in the mitochondria of lower eukaryotes (Attardi, 1985). Mammalian mitochondrial tRNAs are generally smaller than their cytoplasmic and prokaryotic counterparts (Cantatore et al.,

1982), and only 22 tRNA genes are found instead of the 32 required to read the genetic code according to the wobble mechanism (Barrell et al., 1980; Cantatore and Saccone, 1987). This genetic code is also different from the universal code, in that it uses a UGA code for tryptophan rather than for termination, and the AUA codes for methionine, rather than the usual AUG (Barrell et al., 1979; Anderson et al., 1981).

DNA and RNA sequence analysis indicates that mammalian mitochondrial mRNAs contain few or no nucleotides as 5' or 3' untranslated leaders. Hence, this system will not use a Shine-Dalgarno sequence type of interaction to specify the start AUG (Ojala, 1980; Attardi et al., 1981). Furthermore, the lack of a 5' leader or cap structure indicates that the mitochondrial ribosome will not use a cap binding and scanning mechanism for initiation such as that found in the eukaryotic cytoplasmic system (Grohmann et al., 1978; Kozak, 1978; Montoya et al., 1981). The pathway for the assembly of the initiation complex in animal mitochondria appears to be quite different from the pathways found in either the prokaryotic or eukaryotic cytoplasmic systems. The small subunit of the mitochondrial ribosome appears to have the ability to bind mRNA tightly in a sequence-independent manner without the need for initiation factors or the initiator tRNA to be bound (Liao and Spremulli, 1990). This interaction requires mRNAs of over 300 nucleotides in length and appears to be independent of the secondary structure of the RNA. The initiation factor, IF2_{mt}, may help to facilitate the localization of the subunit at the 5' end of the mRNA where the start codon is located. Only one 28S subunit is capable of interacting with each mRNA, no matter what the length of the mRNA (the average length of mitochondrial messages is 350 to 1800 nucleotides). This

is possibly due to the mRNA wrapping around the 28S subunit to form a compact structure.

The mitochondrial ribosomes are small, about two-thirds the size of *E. coli* ribosome, and composed of only 25-30% rRNA (Matthews et al., 1982); whereas eukaryotic ribosomes are 45-50% rRNA, and *E. coli* ribosome is 70% rRNA (Cammarano et al., 1972; Van Holde and Hill, 1974). The bovine mitochondrial ribosome is a helpful model system (Denslow and O'Brien, 1979; Matthews et al., 1982). Its ribosome contains 85 proteins, about twice as much protein as RNA. The molecular weight of the proteins is larger than *E. coli*'s. but similar to bovine cytoplasmic (Matthews et al., 1982). There are less than 100 ribosomes per mitochondrion; eukaryotes have about 25,000 cytoplasmic ribosomes per cell (Cantatore et al., 1987). In addition, it has been shown that animal mitochondrial 28S subunits have a binding site for guanine nucleotides and it is possible that a subunit bound GTP may participate in the assembly of the initiation complex in this organelle (Denslow et al., 1989). No 5S rRNA has been found in the metazoan mammalian mitochondrial ribosomes. However, a 23 nucleotide sequence is present at the 3' end of the human mitochondrial 16S rRNA molecule that exhibits 68% similarity in sequence and structure to a portion of the *B. subtilis* 5S rRNA. This could possibly represent a truncated 5S rRNA which has become part of the 16S rRNA gene (Nierlich, 1982; Attardi, 1985).

Mammalian mitochondrial IF2

Bovine mitochondrial initiation factor 2 (IF2_{mt}) has been identified and characterized. The predicted mature form consists of 698 amino acid residues, and has a molecular weight of 78,129. Human and bovine mitochondrial initiation factors appear to be similar in size (see Appendix D). The predicted mature form of the human mitochondrial initiation factor is a monomeric protein with a molecular weight of 77,500, which encodes a protein of 698 amino acids. The peptide sequence of bovine IF-2_{mt} exhibits 85% identity to human IF-2_{mt}. cDNAs for the human mitochondrial initiation factor have been cloned and characterized from human liver, heart, and fetal brain cDNA libraries (Fig. 6).

MTIF2

Human mitochondrial translational initiation factor 2 is a nuclear gene whose product functions as one of the initiation factors in the process of translation in the mitochondrion. The genomic copy of the gene has been cloned and its DNA is currently being sequenced in Dr. Farwell's lab. The protein that is expressed from this gene is synthesized in the cytoplasm and imported into the inner mitochondrial matrix, where it interacts with molecular chaperones that facilitate its final folding. There it participates in mitochondrial translation.

Fig. 6. Nucleotide and deduced amino acid sequences of human liver IF2_{mt} (Ma and Spremulli, 1995).

Proposal and Background

Locating the MTIF2 gene on a human chromosome may help us to better understand the evolution of protein synthesis in the mitochondria. Mitochondria require proteins that are synthesized in the cytoplasm and then imported. It may be that over evolutionary time, the nuclear genome has acquired genes from the mitochondrion. Mapping this gene will allow us to compare its position to the position of other mitochondrial proteins. Translational initiation represents the focal point of many posttranscriptional regulatory mechanisms. A thorough understanding of its molecular basis and the identification of the determinants of its fidelity and efficiency are prerequisites for understanding regulation of gene expression. This study will also contribute to the current international effort to map and sequence the human genome.

The first mapping of a gene to a specific chromosome was performed in 1911. The gene for color blindness was found on the X chromosome. This led to the discovery over the next 57 years of other X-linked traits such as hemophilia and Duchenne muscular dystrophy. Then the first assignment of a gene to a specific human autosome was made in 1968; the Duffy blood group was mapped to chromosome 1. This led to the feasibility of assigning specific genes to specific chromosomes, or regions of chromosomes, by interspecies somatic cell hybridization (Weiss and Green, 1967).

In 1970, site-specific restriction endonucleases became the scalpel for dissecting the human genome. Cloning of DNA segments in *E.coli* were developed by the laboratories of Boyer, Cohen, Berg and their colleagues in 1972. Southern developed gel-transfer hybridization for displaying restriction fragments of DNA in 1975. In 1977,

two methods of DNA sequencing (Maxam and Gilbert, 1977; Sanger et al., 1977), greatly facilitated mapping to the level of individual nucleotides. In 1981, Sanger's group published the complete nucleotide sequence of the human mitochondrial chromosome. Since 1980 direct methods of human chromosome mapping, such as *in situ* hybridization of radiolabeled or immunofluorescence-labeled DNA sequences to chromosome spreads, have been used to identify the localization of genes on the chromosome (Gerhard et al., 1981; Harper et al., 1981; Szabo and Ward, 1982). By technology of recombinant DNA (Watson et al., 1983), libraries of cloned DNA fragments from the entire human genome were prepared. With reverse transcriptase, cDNA clones of human genes were prepared from the corresponding messenger RNA genes. And in 1986 Mullis and his co-workers invented the polymerase chain reaction.

In order to find the location of the MTIF2 gene, I relied on previous work completed in Dr. Farwell's lab. The complete MTIF2 cDNA was radiolabeled and used to probe a human leukocyte genomic library. Nine positive clones were identified and purified. Restriction digestion followed by Southern blotting using the cDNA probe confirmed eight positive subclones. Several of the genomic clones were used in determining this gene's location on a human chromosome. PCR (polymerase chain reaction) and FISH (fluorescence *in situ* hybridization) were the two techniques I employed in the process. PCR was primarily used for screening the Somatic Cell Hybrid Panel, which is described below. This allowed a general location of the gene to be found. Specific primers for the PCR were designed from the sequence information obtained on genomic clones. One of the primers flanked an intron-exon junction in the 3'-end of the

gene, making it specific for the human mitochondrial initiation factor. Afterwards, to confirm the location, and to give a more exact position on the chromosome, FISH was utilized, with several clones combined and used as one probe. The gene was then assigned a chromosomal position by G-banding. Both techniques are described in Materials and Methods.

MATERIALS AND METHODS

Somatic Cell Hybrid Panel

The human genetic mutant cell mapping panel consists of DNA isolated from 24 human/rodent somatic cell hybrids each retaining one intact human chromosome. It also includes DNA samples isolated from human cell culture, a Chinese hamster cell line, and a mouse cell line. It is utilized as the template DNA in a PCR reaction.

It is possible to fuse one cell with another to form a combined cell with two separate nuclei, called a **heterocaryon**. A suspension of cells is treated with certain inactivated viruses or with polyethylene glycol (PEG), either of which alters the plasma membranes of cells in a way that induces them to fuse with each other. Heterocaryons provide a way of mixing the components of two separate cells in order to study their interactions. The inert nucleus is reactivated to make RNA and eventually to replicate its DNA when it is exposed to the cytoplasm of a growing tissue-culture cell by fusion. Although mouse and human cell-surface proteins are initially confined to their own halves of the heterocaryon plasma membrane, they quickly diffuse and mix over the entire surface of the cell. The heterocaryon will proceed to mitosis and produce a **hybrid cell** in which the two separate nuclear envelopes have been disassembled, allowing all the chromosomes to be brought together in a single large nucleus. For unknown reasons, mouse-human hybrid cells predominantly lose human chromosomes. These chromosomes are lost at random, giving rise to a variety of mouse-human hybrid cell lines, each of which contains only one or a few human chromosomes. This phenomenon

has been put to good use in mapping the locations of genes in the human genome. The same hybrid cells can also be used as a source of human DNA for preparing chromosome-specific human DNA libraries.

Coriell Cell Repository

A human/rodent somatic cell hybrid panel, the Coriell Cell Repository Mapping Panel #2 – Version 3 from the National Institute of General Medical Sciences (NIGMS), Camden, NJ, was used as a source of DNA. It contained 24 hybrid DNA samples. Each sample retained one intact human chromosome. The panel represented the 22 autosomes, plus the X and the Y chromosomes. There was no preparation required with the samples.

Polymerase Chain Reaction (PCR)

The polymerase chain reaction technique was developed in 1986 by Kary Mullis and colleagues while working in the Department of Human Genetics at Cetus Corporation. It relies on the fact that the enzyme DNA polymerase requires a double-stranded primer section of DNA in order to initiate DNA synthesis. A special DNA polymerase from a species of bacteria that lives in hot springs and is stable at temperatures of 90°C is used. In the PCR technique, that primer is supplied in the form of a synthetic oligonucleotide, allowing the replication of a specific region of DNA. DNA polymerase can be directed to replicate only one specific region from an entire genome. Because these same primer sites are copied onto the newly synthesized strands of DNA, after each round of replication they can be used as primers. By repeating the

replication process, a region of DNA can be amplified millions or billions of times, producing enough copies of the DNA for experimental purposes without the need for cloning. The DNA to be amplified does not have to be purified, and can come from any number of sources, including genomic DNA, dried blood, single hairs, fossils, etc.

In Mullis's procedure the double-stranded DNA was dissolved in a polymerase buffer. The four dNTPs were added, along with the two oligonucleotide primers. A single cycle was performed by heating the reaction to 95°C for 2 min, cooling to 30°C for 2 min, then adding 1 unit of Klenow fragment from *E.coli* DNA polymerase I. The extension reaction was allowed to proceed for 2 min at 30°C. The cycle was terminated, and a new cycle initiated by returning the reaction to 95°C for 2 min. The cycle was repeated ~25 times (Mullis et al., 1986). The general procedure, therefore, involves three steps. First, heating the DNA to about 90°C until it dissociates into single strands (usually about 5 min). The second step is the annealing of primer sequences to the single strands of DNA. Two different primers are most often used in PCR. Each primer has a sequence complementary to only one of the two strands of DNA. The primers align themselves with their 3' ends facing each other since they anneal to opposite strands. The third step is the actual amplification event, carried out by extension of the primers using single-stranded DNA as a template and thermostable DNA polymerase to extend the oligonucleotide primers in the 5' to 3' direction. The three steps comprise a cycle. Twenty to twenty-seven cycles result in a several-million-fold increase in the amount of target DNA. The process is automated by use of a thermocycler. The results of the PCR are usually tested by gel electrophoresis.

PCR primers

PCR primers are short oligonucleotide sequences, usually 20-25 base pairs, which are designed using DNA sequence information. Each primer has a sequence that is complementary to only one of the two strands of DNA. This allows the replication of a specific region of DNA. If the primers contain sequence information from both an exon region and an intron region of a particular gene, they will be more specific to locating and hybridizing exclusively to that gene since introns are not likely to be conserved even among gene homologs.

PCR method

To amplify the DNA of the somatic cell hybrid panel the following reaction mixture was used. Primers were obtained from Gibco/BRL in 100 μ M concentrations, and brought to a final concentration of 10 μ M with 1X TE, pH 8.2. The forward and reverse primers, respectively, were: 'E': (5'-GGGAACCCATCAACTACAGAC-3') and 'H': (5'-GGACATGTAATTTGGAAGGG-3'). Individual chromosome DNA was used as the template. Each concentration was approximately 0.35 mg/ml. A 50 μ l reaction volume was prepared for each sample. 28 samples were prepared and a molecular weight standard (100 bp ladder ranging from 100 to 1000 bp). These included the 24 DNA samples from the hybrid panel, the human parental DNA as a positive control, a negative control with just primers and no DNA, and the mouse and Chinese hamster parental DNAs. Tubes were placed on ice, and materials added in the following order: 45 μ l PCR SuperMix (Gibco/BRL), which contained 22mM Tris-HCl, pH 8.4, 1.65 mM $MgCl_2$,

55mM KCl, 220 μ M dNTPs and 27 U recombinant Taq DNA polymerase/ml; 2 μ l of each of the primers, and 1 μ l of the template. The samples were mixed, and then centrifuged. 30 to 40 μ l of mineral oil were layered on top to prevent evaporation. A thermocycler (M.J. Research Mini Cycler) was then programmed for an initial denaturation of 1 min at 94°C, 35 cycles of 40 sec at 92°C, 60 sec at 55°C, 2 min at 72°C, and a final extension at 72°C for 5 min. Products were held at 4°C until electrophoresed on a gel.

Electrophoresis

A 95 ml (10 mm x 14 mm, 6 mm thick) 3% intermediate melting agarose gel (Metaphor, FMC BioProducts) was prepared in 1X TAE, pH 7.8. Based on its intermediate melting properties, the set up time for the gel was 30 min at RT, then 30 min at 4°C. The running buffer was 850 mls of 1X TAE, pH 7.8. Twenty μ l of each sample were mixed with 4 μ l of a dual tracking dye (0.05% each xylene cyanol and bromphenol blue in 20% sucrose). A Gibco/BRL Model H5 Horizontal Gel Electrophoresis Apparatus was used for the gel. Twenty-four μ ls were loaded in each well. A gel has the capacity for 14 wells only, so three gels had to be set up. One gel had the negative control, the positive control, the mouse and hamster preparations, and the first seven chromosome samples. The second gel had molecular weight standard and chromosome samples 8 through 18. The third gel had a molecular weight standard and the remaining 6 chromosome preparations. The gels were electrophoresed at 100V for 2 hours. After electrophoresis the gels were stained with 42.5 μ l of ethidium bromide (0.5 μ g/ml final concentration) for 30 to 60 minutes.

They were viewed on a UV illuminator box (Fisher Biotech Electrophoresis Systems, 312 nm Variable Intensity Transilluminator), photographed using a Kodak Digital camera and processed using the Kodak Digital Science 1D software, version 1.6.

Fluorescence *In-Situ* Hybridization (FISH)

By definition, *in-situ* hybridization is the annealing of RNA molecules to their complementary DNA molecules. The resulting molecular hybrids are generally detected by scintillation counting of radioactive RNA after treatment with ribonuclease to remove unhybridized RNA (Hsu, 1979). Better hybridization and detection techniques were developed in the 1970's, and fluorescence *in situ* hybridization was first used to fluorescently label specific nucleic acid sequences in cells or chromosomes in the early 1980's (Langer-Safer et al., 1982; Landegent et al., 1985; Hopman et al., 1987).

Fluorescence *in situ* hybridization involves hybridization of biotin- or digoxigenin-labeled DNA probes to denatured metaphase chromosomes and visualization of those probes with fluorochrome-conjugated reagents. Hybridization today employs DNA as probes of genomic material for the purpose of gene mapping. Probes can be labeled either radioactively or chemically, and detected by a variety of methods (Pinkel et al., 1986). Chemically modified probes are more advantageous because the hybridization can be detected with reagents that bind with high affinity to the probe, and carry various types of reporter molecules, such as fluorescent dyes. Useful fluorochromes for FISH include the blue fluorochrome AMCA (amino-methylcoumarin-acetic acid), the green fluorophore fluorescein, and the red fluorochromes CY3, rhodamine, and Texas Red™.

Dyes can provide quantitative analysis through intensity measurements and high-resolution mapping. Pinkel's procedure was a modification and simplification of the procedures developed by Mary Harper (Harper et al., 1981) and David Ward and colleagues (Langer-Safer et al., 1982). It involves applying a fluorescein-labeled avidin antibody (reporter-binding molecule) to the hybridized chromosome preparation containing biotin (reporter molecule). Every avidin molecule contains four biotin-binding sites. For signal amplification, a biotin-labeled antibody to the avidin is added. The avidin antibody can potentially bind the avidin molecule by a biotin-avidin interaction or an antibody interaction. Another application of fluorescein-conjugated avidin results in significant signal amplification. Finally the chromosomes are counterstained with propidium iodide. This allows the hybridization signal to be seen as a bright yellow-green dot on an orange-red-colored chromosome when viewed on an epifluorescent microscope with the appropriate filter. Once the gene is mapped to a general chromosome section, a more precise location can be determined by G-banding.

Slide making for the hybridization

Preparation of slides. Metaphase chromosomes were obtained from normal human peripheral blood lymphocytes by standard techniques (East Carolina University School of Medicine Cytogenetics Laboratory). The fixed cells were dropped onto clean wet cold slides, from a height of about 12 inches to break open the cell. The slides were flooded with a 3:1 methanol: acetic acid solution to fix the spreads to the slides, and placed over steam for 20 to 40 sec (depending on the room humidity). Quantity and quality of

metaphase spreads were assessed with phase contrast on an inverted microscope.

Conditioning and Denaturing of slides. Slides were conditioned/aged in 2X SSC (0.6 M NaCl, 0.06 M sodium citrate) at 37°C for 30 min if they were less than 3 days old. The slides were then dehydrated in an alcohol series (70%, 85%, and 100%) at room temperature for 2 min. each, air dried, and placed in a 70% formamide/2X SSC solution at 70°C for 2 min. to denature the chromosomal DNA. The alcohol dehydration and air drying steps were then repeated, but with cold 70% ethanol.

DNA preparation

Because the insert size of each clone was 18 kbp, several clones (2-2, 8-1, J-1, and J-2) were combined in a “cocktail” for use in the hybridization. Each probe DNA was individually biotin labeled by nick translation (Langer et al., 1981; Langer-Safer et al., 1982) with Biotin-14-dCTP (Enzo) using the following procedure. One-ml microfuge tubes were placed on ice. 1 µg of the each clone DNA was added to a separate 50 µl reaction mixture of 10% Soln A (0.5 M Tris at pH 7.8, 50 mM MgCl₂, 0.5 mg/ml Bovine Serum Albumin, nuclease free), 10% Soln B (0.5 mM dNTPs), 10% Soln C (0.1 M 2-Mercaptoethanol), 10% Biotin-14-dCTP, 5% DNase/Pol I mixture, and water to volume. The mixture was incubated at 15°C for 90 min, after which the reaction was stopped and the probe precipitated by adding 15 µl 3M NaAc/µg DNA. For every µg of DNA, 2 µl of Paint Pellet (Novagen) was added for tracking purposes. The samples were mixed, 300 µl cold 100% EtOH added to each, then they were placed in a -20°C freezer overnight. The next day the samples were cold centrifuged on a Baxter Biofuge A for 15 min at

13,000 rpm, placed on ice 10 min, and cold centrifuged again 15 min. The supernatant and washes from all steps were removed and saved for the Dot Blot procedure. The pellet was washed 2 times with cold 70% EtOH before the centrifuge steps were repeated. The probe DNA was then washed with cold 100% EtOH, and dried in a vacuum dessicator overnight. Dry probe was resuspended in 25 μ l 1X TE/ μ g, pH 8.0, and incubated for 15 min at 37°C. The resulting probe concentration was approximately 40 ng/ μ l.

Detection of nick translation of probe DNA

This procedure was used to check the quantity and quality of the biotin labeling by comparison to a known biotinylated standard DNA sample. The supernatant and the 70% and 100% ethanol washes from the nick translation steps were dried in a dessicator overnight. They were rehydrated the next day in 10 μ l of 1X TE, pH 7.5. Dilutions (1/10, 1/ 100, 1/1000) of the biotinylated samples and the supernatant and washes from the procedure were made in sterile distilled water. On a scored nitrocellulose membrane, 1 μ l of each sample and dilution was deposited in separate locations and air-dried. Standard markers (200 pg/ μ l biotinylated lambda DNA, 0.2 μ g/ μ l sheared herring sperm DNA in 6X SSC) were also applied in full strength and the same dilutions as the biotinylated sample. The membrane was then moistened from behind with a few drops of 2X SSC. When completely wet, it was drained thoroughly and placed in a Stratalinker 1800 (Stratagene) for 30 sec to bind the DNA. Afterwards the membrane was soaked for 1 min in a plastic container with Gibco's BluGene buffer 1 (0.1M Tris-HCl, pH 7.5,

0.15M NaCl) to correct the pH and salt conditions. It was then incubated in a 37°C water bath for 1 hour using a plastic container filled with BluGene buffer 2 (3% (w/v) bovine serum albumin in buffer 1) for blocking. For the detection, a stock solution (1.0 mg/ml in 3M NaCl, 1mM MgCl₂, 0.1mM ZnCl₂) of streptavidin/alkaline phosphatase (BRL) was diluted to 1µg/ml with BluGene buffer 1. 7 µl of the buffer per 100 cm² of membrane were added to a plastic bag along with the membrane, bubbles excluded, and the bag sealed. It was incubated at room temperature for 10 min on a shaker platform. Then the membrane was removed to a fresh plastic container and washed with Blue Gene buffer 1 for 15 min on a shaker. The wash was repeated again, followed by a wash with BluGene buffer 3 (0.1M Tris-HCl, pH 9.5, 0.1M NaCl, 50 mM MgCl₂) for 10 min. The membrane was slightly drained, then sealed in another plastic bag with freshly made NBT/BCIP dye solution (nitroblue tetrazolium; 75 mg/ml in 70% dimethylformamide; 5-bromo4-chloro-3-indolylphosphate: 50 mg/ml in dimethylformamide) and placed in a lightproof box. It was incubated for 3-5 hours, removed from the bag and washed in BluGene finishing buffer (20mM Tris, pH 7.5/0.5mM Na₂EDTA) for 5 min to remove excess background. After drying between sheets of filter paper #3, the membrane was checked for appearance of dots, signifying biotinylation. Quantitation was accomplished by comparing the size of the dots to standard markers, which were applied at the same time.

Suppression of repeated sequences

Cot-1[®] human placental DNA (Gibco/BRL) was used to suppress repetitive sequences

found in the probe (Landegent et al., 1987; Lichter et al., 1988b; Pinkel et al., 1988). Salmon Testes DNA (Sigma) was used to suppress repetitive sequences found in the human genomic DNA of the metaphase spreads on the slides. A 20-fold (10 µl) addition of Cot-1[®] DNA (1 mg/ml) was mixed with 8 to 12 µl (200-500 ng) of the biotinylated probe and lyophilized using a SpeedVac vacuum centrifuge for 30 min at 15,000 rpm and 30°C. The pellet was resuspended in a solution of 8.6 µl of 50% formamide/2X SSC and 1.4 µl of Salmon Testes DNA, 11 mg/ml (final concentration of approximately 500 ng/µl). The sample was then mixed, centrifuged and placed in a 37°C water bath for 30 min to rehydrate the lyophilized DNA. The preparation was denatured for 10 min at 70°C. This temperature depends on the melting temperature of the probe DNA, and can vary from 60 to 90°C. Sequence data for my probe suggests an A-T rich combination. The following formula (Bolton and McCarthy, 1962) is used to predict the correct denaturing temperature to use: $81.5^{\circ}\text{C} - 16.6(\log_{10}[\text{Na}^{+}]) + 0.41(\% \text{G-C}) - 0.63(\% \text{formamide}) - (600/\text{length of hybrid in bp})$. After denaturing, in order for the Cot-1[®] to suppress the repetitive sequences, a preannealing step was carried out at 37°C for 30 to 60 min in a water bath before hybridization.

Hybridization

A hybridization mixture (50% formamide, 20% dextran sulfate in 2X SSC, pH 7.0) was prewarmed to 37°C, then 20 µl was added to the DNA, mixed and centrifuged (total volume 30 µl). Since the recommended volume per slide for hybridization is 2.5 µl per

cm² and a 24 x 50 mm coverslip was used (area is 12 cm²), the volume used was 30 µl. All 30 µls were transferred to a prepared slide and the glass coverslip added. The edges were sealed with rubber cement. The slide was then placed on a raised platform in a humid chamber (a Tupperware container with a towel wetted with 2X SSC in the bottom). The chamber was placed in a 37°C incubator overnight for at least 16 to 18 hours.

Post-hybridization

After incubation, the rubber cement was removed with forceps and the coverslips floated off in a 1X PBD (phosphate buffered detergent) solution for about 30 min. The slides were washed 2 times in 50% formamide/2X SSC, pH 7.0, for 10 min at 43°C, then in 2X SSC, pH 7.0, 37°C for 10 min, and once in 2X SSC, pH 7.0, room temperature for 10 min. A final wash and hold step was done in 1X PBD, room temperature, until ready for the detection steps.

Detection

After the 1X PBD rinse, the slides were allowed to drain but not dry and 60 µl of a blocking buffer were applied. Each slide was then covered with a plastic coverslip and incubated for 5 min at room temperature under low light conditions. The reagents for the detection of the biotinylated probes were all obtained from Oncor. Coverslips were removed, slides drained briefly, and 60 µl fluorescein-labeled avidin applied. Slides were recovered with plastic coverslips and placed in a humidified chamber at 37°C for 20 min.

Slides were washed three times in 1X PBD buffer for 2 min each, then drained. 60 µl of anti-avidin antibody were added, coverslips applied, and incubated another 20 min at 37°C in a humidified chamber. The 1X PBD washes are repeated, slides drained, and 60 µl of Fluorescein-Avidin applied. The 20 min incubation at 37°C was repeated. The slides were washed again in 1X PBD for 2 min each, drained, and 20 µl propidium iodide/antifade and 10 µl antifade (premixed in a light-safe tube) added. A glass coverslip was applied to each slide. The slides were then stored in a lightproof box and placed in a -20°C freezer for at least 20 min before viewing on the microscope.

Metaphase analysis. At least 100 metaphases were examined on a Zeiss Axioplan epifluorescence microscope to establish the location of the gene. The number of signals was counted in each spread. Ideally 4 signals would be seen in each spread, one on each chromatid. However, due to chromosome positioning or weak hybridization that is not always possible, and sometimes only one to three signals were seen. High quality metaphases were photographed using Kodak Gold 400-speed color film.

G-banding technique

G- banding is a type of staining for chromosome preparations named after Gustav Giemsa, who invented the stain in the early 1900's to stain malaria blood preparations. It is a mixture of azure, eosin, glycerin, and methanol in a buffer solution. Chromosome banding has been in use for about 30 years and encompasses a great variety of techniques. According to the Paris Conference (1971), a chromosome band is part of a chromosome that can be distinguished from adjacent segments by appearing darker or

lighter. Q bands are produced by quinacrine-staining methods, and Giemsa staining produces G bands. R bands are the reverse of Q and G bands and are produced by fluorescent and nonfluorescent R-staining methods. T bands are located at the telomeres, and C bands are the constitutive heterochromatin found at centromeres. And finally, N bands are nucleolar organizer regions produced by silver-staining methods (Barch, 1991).

Conventional Giemsa staining allows location of the centromere, measurement of the short: long arm ratio, detection of large structural abnormalities, and the identification of only a few chromosomes by size and morphology. In 1968, Caspersson and colleagues introduced quinacrine mustard to plant cytogenetics, and in 1971 he and his colleagues published the photometric fluorescence patterns (Q-banding) for the 24 different human metaphase chromosomes. The ability to uniquely identify each chromosome by its characteristic banding pattern has been of fundamental importance to gene mapping (Caspersson et al., 1970; Patil et al., 1971; Schnedl, 1971; Seabright, 1971; Sumner et al., 1971). Banding methods applied to metaphase chromosomes could demonstrate about 400 bands in the total karyotype; high-resolution cytogenetics as developed by Yunis (1976) and others for application to extended chromosomes in prophase or prometaphase could demonstrate more than twice that number (McKusick, 1986). In Q and G bands it has been suggested that the dark bands represent late-replicating regions which are usually high in AT content and the light bands areas high in GC content (Bernardi et al., 1985; Goldman et al., 1984). However, base composition is not the only factor involved in the production of banding patterns.

Quinacrine banding can be difficult, requires expensive fluorescence equipment,

and is subject to fading. Giemsa banding was introduced in 1971 by Sumner and colleagues and has become an invaluable tool in the cytogenetics lab. The same effect as Giemsa staining can also be achieved using Wright's Stain (1.5 g Wright Stain/500 ml absolute methanol, filtered), which I employed for my procedure. Slides used from the hybridization procedure are prepared then stained with Wright's Stain/Gurr buffer, pH 6.8 (1:4) solution. They are viewed by bright-field microscopy, with the proper filter. A gray and white banding pattern is observed and photographed with Kodak black and white 25-speed film. Photographs of the same spreads taken of the chromosomes with the FISH hybridization signals and the G-banded chromosomes are compared to give the exact chromosomal location according to the banding pattern.

G-banding

Coverslips were soaked off in 1X PBD for 30 min, then the slides were dehydrated in an alcohol series (70%, 85%, and 100%) for 2 min each and air-dried. The slides were immediately rinsed twice with isotonic saline solution, drained briefly and placed horizontally. Wright's Stain (1.5 g/500 ml absolute ethanol) mixed 1:4 with Gurr buffer, pH 6.8, was used to flood the slides for 2 min. After rinsing with distilled water, the slides were air-dried. They were examined on a Zeiss bright field microscope, with a green filter, and G-banded metaphases were photographed with Kodak Panchromatic 25 speed black and white film. Comparison of the bands with the hybridization site allowed for determining a precise location of the gene on the chromosome.

RESULTS

The results seen in Figure 7 are from the PCR reaction that was electrophoresed on an agarose gel. Two bright bands are seen on the gel; one representing the amplified DNA from chromosome 2 and the other from the human parental sample, which was used as a positive control. Part of the molecular weight standard is shown, each bright band representing 100, 200, 300, and 400 base pairs. The size of the both bands was approximately 200 bp, as anticipated. None of the other lanes showed any bands.

The confirmation of the gene's location on chromosome 2 was provided by the FISH procedure. Examination of the stained and labeled hybridization slides with an epifluorescent microscope revealed several metaphase spreads with signal. Figure 8 shows the orange-red chromosomes with a fluorescent yellow-green dot on each chromatid where the hybridization has occurred. It gives the appearance of two "snake eyes" glowing. About 60 good quality metaphase spreads, free from cytoplasm and with chromosomes randomly dispersed were counted. Thirty spreads showed 4 hybridization signals, one on each chromatid of each chromosome 2. Nineteen spreads showed 3 signals, ten spreads showed 2 signals, and one spread had only one signal. Four signals are not always seen due to the placement of the chromosome on the slide; that is, the chromosome can be bent, folded or turned in the area of the hybridization signal, and the signal can be obscured from vision.

G-banded metaphase chromosomes seen in Figure 9 were compared to the FISH photographs for location of the hybridization signal. Measurement of the distance of the signal on chromosome 2 was transferred to the G-banded chromosome to give an exact

location as described in Materials and Methods. Figure 10 is an ideogram of chromosome 2 showing the map location of MTIF2.

In summary, the MTIF2 gene location was mapped to human chromosome 2. This result was obtained using three different techniques. The general location found, using the PCR technique and the Coriell panel of somatic cell hybrids, was on chromosome 2. That location was confirmed by fluorescence in situ hybridization (FISH) to the short arm of chromosome 2. It was further localized, by G-banding, to the area 2p16 to 2p14 (Bonner et al., 1998).

Fig. 7. Composite of agarose gels showing PCR of somatic cell hybrids. The lanes are numbered for the human chromosome present in that hybrid. N = no DNA; H = human genomic; M = mouse control; C = Chinese hamster control; S = standard marker.

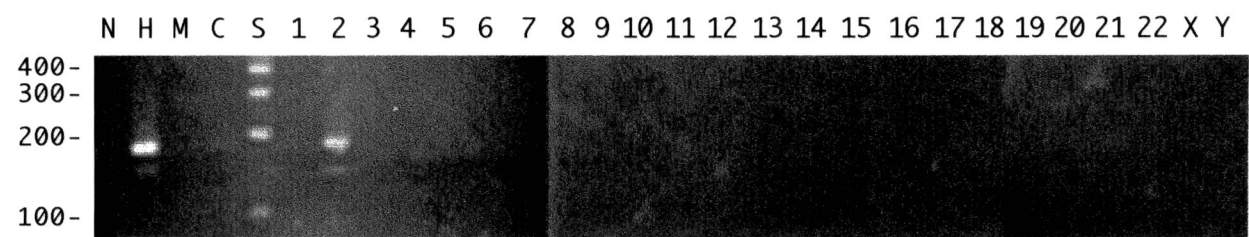


Fig. 8. A human metaphase spread hybridized to the probe displaying signals on both chromosome 2 homologs at 2p16→p14.

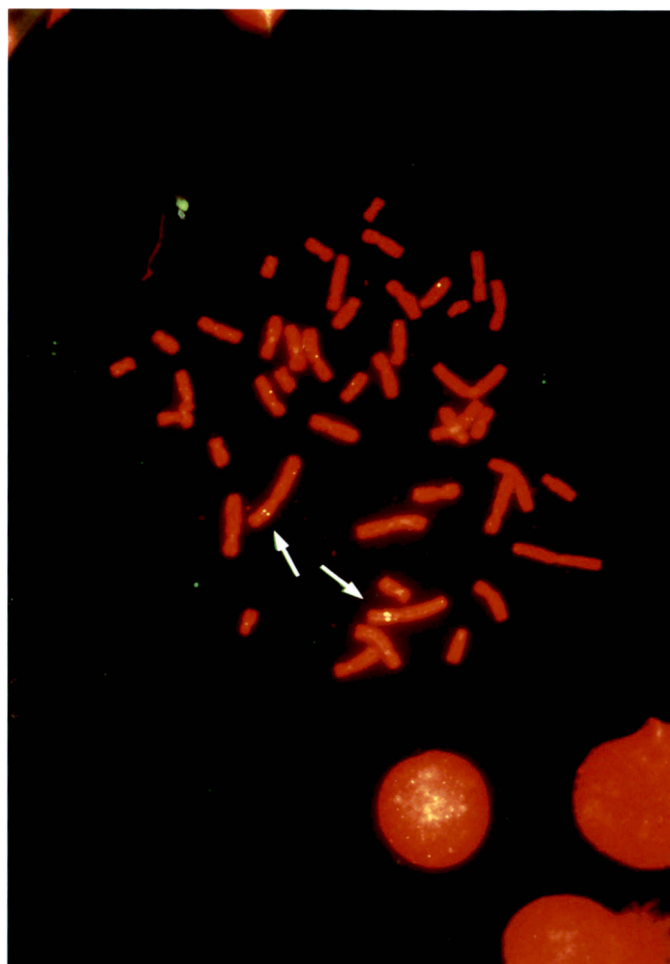
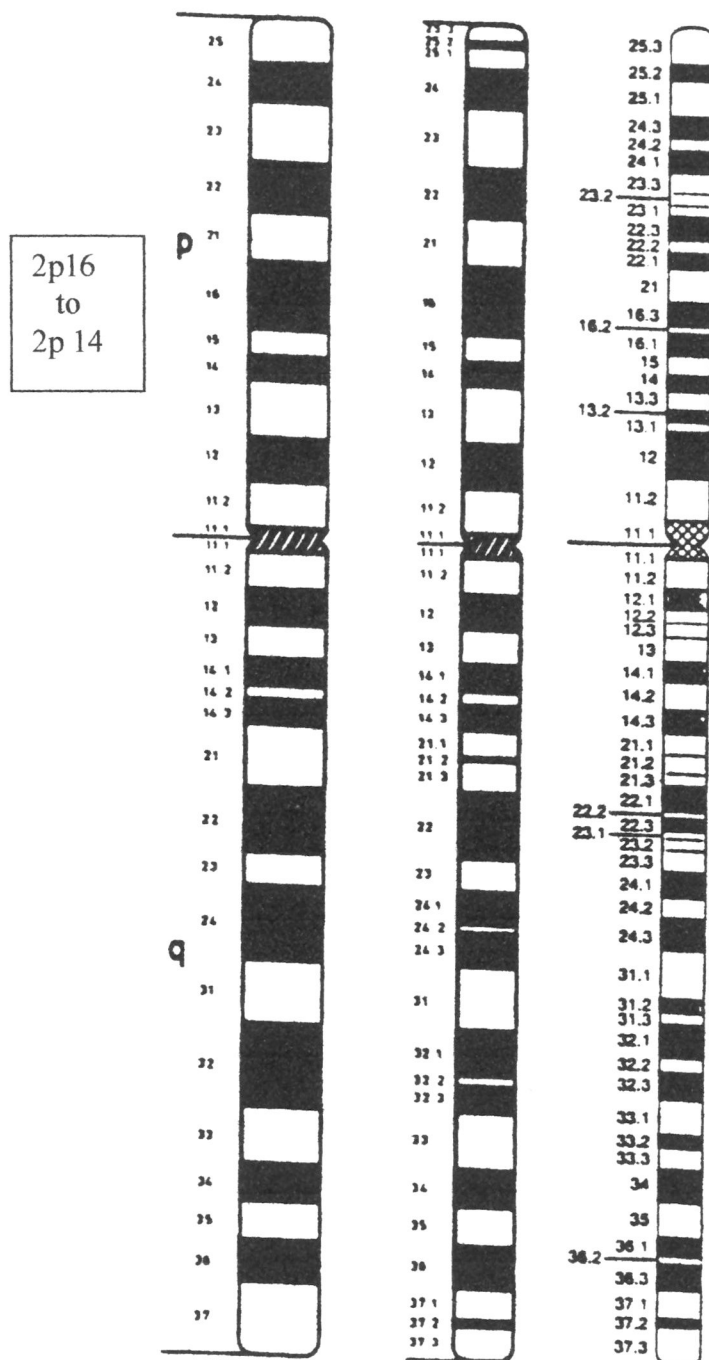


Fig. 9. G-banded metaphase chromosomes.



Fig. 10. Ideograms of G-banding patterns for normal human chromosomes at three different levels of resolution. The left chromosome represents a haploid karyotype of approximately 400 bands, the central ideogram is at the 550 band level, and the 850-band ideogram is at the right. The location and width of bands are not based on any measurements. These ideograms correspond to the ISCN (1981) nomenclature (Mitelman, 1995).



DISCUSSION

Although it appears straightforward, mapping a gene to a human chromosome entails much work and some luck. The subclones and primers were acquired from Dr. Farwell's lab from previous research work. The PCR technique had already been automated by development of a thermocycler. The use of a somatic cell hybrid mapping panel eliminated the need to make DNA hybrids from scratch. Several trial runs were made using the primers and a lambda genomic clone, which contained MTIF2 DNA sample. Various samples of DNA from the mapping panel were used and success was achieved. A positive result for chromosome 2 and the human parental was attained. DNA representing the remainder of the chromosomes and the parentals from the mouse and Chinese hamster were then tested. The results were negative, implying that the gene was not located on them. The PCR reaction was repeated several times to confirm the results. Attention then turned to optimizing the conditions for maximal PCR efficiency. This is where the learning and work began.

First, the PCR buffer volume conditions were questioned. Was a standard 50 μl final volume necessary? Thirty-five, forty, and forty-five μl were tested, varying just the Super Mix buffer solution, without changing the primer or DNA amounts. The volume of the final reaction appears to be critical, because under 45 μl , the reactions were all negative. Fifty μl of reaction mixture was agreed upon as the final volume.

It is known that the presence of divalent cations is critical to primer template fidelity and primer extension. The PCR enzyme Taq polymerase needs magnesium. However, the concentration of free Mg^{2+} decreases as the level of dNTPs increase.

dNTPs are the major source of phosphate groups in the reaction and changes in their concentration affects the concentration of available Mg^{2+} because the dNTPs chelate a proportional number of Mg^{2+} ions (Chien et al., 1976). It was necessary to know the effects that varying the levels of magnesium would produce. SuperMix-like buffer solutions were prepared in the range of 1.4 mM to 1.6 mM. Results for the PCR reaction were electrophoresed on an agarose gel. The strongest band signal for chromosome 2 was from the 1.4 mM concentration. However, the human parental sample had a preference for the 1.6 mM concentration. An average of 1.5 mM level was an acceptable compromise. Since the commercial SuperMix (Life Technologies) contained that amount, the remaining tests would use the SuperMix as the buffer solution.

Next, the operating parameters for the PCR reaction were varied from 90°C to 95°C and from 3 to 7 minutes for the denaturation step. Higher temperatures and longer times gave negative results, and it was concluded that they led to the degradation of the DNA. Annealing temperatures and times were varied from 45°C to 65°C, with times of 30 sec to 2 min; and the extension times were varied from 1 min to 3 min. A final extension time at the end of all the cycles was varied from 3 min to 15 min, but didn't seem to have an effect. The optimal parameters were chosen and are reflected in the Methods and Materials section.

In choosing a proper agarose type and concentration for the electrophoresis gel, standard high-melt agarose was chosen as the first option. 0.7% to 2% concentrations were used, but the bands produced were fuzzy. Research of the manufacturing catalogs suggested using a low or intermediate melt agarose. Both were tried in varying

concentrations. The low melt agarose was extremely difficult to work with, since it never gelled at room temperatures (24°C). The intermediate melt agarose was tried at different concentrations suggested by the manufacturer. A 1%, 2%, 3%, and 4% gel was prepared, and samples run. The higher the concentration, the more difficult it was for the agarose to go into solution. Although the 4% gave the cleanest, clearest band, the difficulty in preparation eliminated it as an option. The 3% concentration was chosen as having the optimal characteristics for preparation, run time, and band appearance. Using all the chosen parameters, the PCR reaction was again performed, and the products electrophoresed on the 3% intermediate melt agarose gel. These results are seen in Figure 7.

The results from the somatic cell hybrid panel had to be confirmed by another method and FISH was chosen for the procedure. The medical school cytogenetics department directed by Dr. John Wiley had the equipment and reagents readily available. One of the clones which had given good results in the PCR reaction was chosen as the probe for hybridization to normal human metaphase spreads, which had been prepared by standard cytogenetic procedures. *In situ* hybridization methods already established for research work on amphibian DNA and clinical work on human DNA were tried first.

Initial attempts to nick translate the probe with dUTP, using the laboratory methods in place, gave poor results. No hybridization signal could be detected, and the background interference was overwhelming. Many attempts at hybridization were tried over a nine month period. Nick translation with dCTP gave better results. The dCTP nicked probe was then used under the same conditions as the dUTP-nicked probed, and

results were slightly improved. A weak signal was inconsistently detected.

Work then concentrated on eliminating the background signal. Suppression of repetitive sequences in the probe was accomplished using Cot-1™ human placental DNA. Background noise started to disappear. Varying the amounts gave better results. Salmon testis DNA was suggested to suppress the repetitive sequences found in the genomic DNA of the metaphase spreads. Also varying the amounts of it had a positive effect. The background was now clear enough to determine if hybridization signal could be located.

At this point it was suggested that the size of the probe might not be large enough to produce a strong consistent signal. Discussion with Dr. Farwell revealed that the other subclones in the lab were overlapping in their sequence data. Dr. Wiley suggested mixing a “cocktail” of several clones to produce a larger probe. I mixed four nick-translated probes together in one reaction, and the results were not strong or consistent, and a lot of background reappeared. Quantity of probe used was questionable, as were the certain characteristics of the reagents used.

Attention was paid to the pH of the different reagents used in the reaction. After testing, it was found that there was inconsistency in their values. All new reagents were prepared and brought to a pH of 7.0. Keeping the reaction mixtures for the hybridization and post-hybridization procedures at the correct pH turned out to be a critical factor. At the same time, calculations were performed to determine the exact quantity of DNA to apply to each slide in the hybridization process. Taking into consideration the varying levels of DNA concentration of each clone, the amounts of suppression agents had to be

adjusted. A literature search revealed the correct percentage of probe to be dependent on the amount of DNA on the slide used in the hybridization. Too much or too little probe can give poor results.

Final attention was given to the post-hybridization washes, considering the stringency and the temperature of the wash, and the amount of time the slides were in contact with the solution. A commonly accepted set of conditions, described in Materials and Methods section, was chosen and worked well. The last step was the fluorescein tagging for detection purposes. Standard amounts were applied, but hybridization times and wash conditions were monitored to achieve the fastest and best results.

Taking all the parameters into consideration, a final experiment was performed and proved to be successful. A classic hybridization signal was obtained, with minimal background interference. Photographs were taken and a sample is shown in Figure 8. Two more repeat experiments were conducted to confirm the results.

It may be concluded from this experience that attention to detail was very important. A new method was developed in the process. Future work with small unique sequence probes should be expedient and successful with minor adjustments.

Other work being done on IF2_{mt} includes a search for pseudogenes and DNA sequencing of the gene. To date, 13 exons have been found and sequenced, and the lengths of several introns have been approximately determined, and some sequence data is available. The 5' end is still unknown, which probably encompasses the regulatory mechanisms. Sequence data is being compared to other initiation factors and to IF2s of other species. Hopefully the structural conformation will soon be deduced, and IF2's

interactions with other initiation factors will be better understood.

Future Work

The location of this gene will help in understanding how the mitochondrion functions. Mapping this gene will allow us to compare its position to the position of other mitochondrial proteins. Initiation represents the focal point of many posttranscriptional regulatory mechanisms. A thorough understanding of its molecular basis and the identification of the determinants of its fidelity and efficiency are prerequisites for understanding regulation of gene expression. Mutations in this organelle have been linked to several diseases: myoclonic epilepsy, ragged red fiber disease, Leber's hereditary optic neuropathy, and Kearns-Sayre syndrome. Furthermore, mitochondria have been linked to neuromuscular disorders and possibly aging (Wallace, 1988, 1992). I hope results from this study will contribute to the current international effort to map and sequence the human genome.

APPENDIX A

Svedberg coefficients

The Svedberg coefficient (S) is a standardized unit that measures the sedimentation behavior during centrifugation. It depends on a molecule's density, mass, and shape. For example, a 5S molecule has a molecular weight of 35,000 and is composed of 120 nucleotides; whereas a 28S molecule has a molecular weight of 1,800,000 and has 4800 nucleotides. The scale is not linear because the size and shape of the molecule impact on its rate of sedimentation.

APPENDIX B

E.coli IF2

Both the α and the β form of *E. coli* IF2 exist in the cell in a 2:1 molecular ratio and are both active *in vivo* and *in vitro* (Hershey, 1987). IF2 γ is formed by proteolytic cleavage of IF2 α during purification (Cenatiempo et al., 1987). The IF2 α protein has six structurally compact and functionally separate domains found among the 890 amino acid residues. Domain I (amino acids 1-103) is found only in the N-terminal part of the IF2 α form. The N-terminus region seems to be species specific and is not related to translation (Gualerzi et al., 1991). Domains II through VI are found in both forms of IF2. Domain II is broken into two regions. The first region (amino acids 104-155) has a very abnormal amino acid composition consisting essentially of only five different amino acids (16 alanine, 16 glutamic acid, 5 lysine, 7 arginine, and 7 glutamine). There is a strict repetition of alanine residues every four amino acids, and the pattern Arg-Glu-Ala is repeated six times. A helical secondary structure is predicted for this region (Sacerdot et al., 1984). The second region (amino acids 167-214) is also rich in alanine, glutamic acid, lysine and arginine. The pattern Glu-Glu-Ala-Arg-Arg is repeated three times. Another helical structure is predicted. Domain III (amino acids 290-391) and Domain V (amino acids 541-671) have unknown functions. Domain IV (amino acids 392-540) is the GTP binding region, containing the 50S and GTP binding sites, as well as the GTPase catalytic site. Residues 404-405 form the beginning of an α -helix whose loop is responsible for the binding of the pyrophosphate group. It exhibits the characteristic

sequence Gly-X-X-X-X-Gly-Lys-Thr, where the lysine is directly involved in the binding of the β -phosphate. Domain VI (amino acids 672-890) is extremely stable under non-denaturing conditions (Laalami et al., 1991). It contains the fMet-tRNA-binding site (Gualerzi et al., 1991). Cells expressing an IF2 protein deleted for the C-terminal quarter of amino acids are not viable, thus demonstrating that this part of the protein is essential (Sperling-Petersen and Mortensen, 1990).

APPENDIX C

Eukaryotic IF2

In eukaryotic translation, protein synthesis is inhibited by phosphorylation of Serine 51 in eIF-2 α subunit, similar to the prokaryotic system (Gaspar et al., 1994). The β -subunit has three unusual polylysine blocks near the N-terminus and a zinc finger motif in the C-terminus, but its function is also unknown. It has been shown to have some GTP binding activity, but it is believed that is due to the γ -subunit's close proximity.

Eukaryotic cells decrease their overall rate of protein synthesis by protein kinases phosphorylating the initiation factor eIF2. Since eIF2 binds very tightly to GDP, a guanine nucleotide releasing protein, designated eIF2B, is required to cause GDP release so that a new GTP molecule can bind and eIF2 can be reused. The reuse of eIF2 is inhibited when it is phosphorylated because phosphorylated eIF2 binds to eIF2B unusually tightly, preventing the completion of nucleotide exchange. There is more eIF2 than eIF2B in cells, and even a fraction of phosphorylated eIF2 can trap nearly all the available eIF2B, thereby preventing the reuse of even the nonphosphorylated eIF2 and greatly slow down protein synthesis. Sometimes the phosphorylation of eIF2 can even enhance the translation of specific mRNAs by shutting down the synthesis of all proteins except those that are required, for example, a missing nutrient. Regulation of the level of eIF2 is also important in mammalian cells as part of the mechanism by which they can be induced to enter a nonproliferating, resting state (G_0) in which the rate of protein synthesis is reduced to about one-fifth the rate in proliferating cells.

APPENDIX D

Mammalian IF2

The bovine factor is 38% identical to the yeast factor, and 39% identical to *E. coli* IF2. Human IF-2_{mt} is 32% identical to yeast and 36% to *E. coli*. Both the bovine and the human initiation factors have a mitochondrial import sequence signal in the first 30 amino acids of the peptide sequence. The N-terminus of either factor shows little identity with the prokaryotic IF2. All three have charged residues at that end, the human factor having 29%, the bovine factor 34%, and *E. coli* 26%. The G-domain is quite conserved in both factors, and they are both 67% similar to *E. coli* IF2. In the C-terminal end, 60% of the bovine factor, but only 50% of the human factor has charged residues. Each of the factors has additional 37 amino acids not found in prokaryotic factors. Similarity in this region decreases between bovine IF-2_{mt} and prokaryotic IF2s, but shows extensive similarity between human IF-2_{mt} and prokaryotic IF2s (Ma et al., 1995; Ma and Spremulli, 1995). The C-terminal domain contains the fMet-tRNA binding site and the G domain contains the 50S and GTP binding sites as well as the GTPase catalytic center (Gualerzi and Pon, 1990).

The bovine full-length cDNA excluding the poly A tail, consists of 2406 base pairs, and is close in size to the mRNA by Northern analysis (~2500 bp). Sequence analysis of the clones showed the mRNA has a 5'-untranslated region of 178 base pairs, a long open reading frame of 2181 base pairs which encodes 727 amino acids, and a 3'-

untranslated region of ~50 base pairs and a poly A tail (Ma et al., 1995).

cDNAs for the human mitochondrial initiation factor have been cloned and characterized from human liver, heart, and fetal brain cDNA libraries. Human IF2_{mt}, like the bovine factor, also has a long open reading frame of 2181 base pairs, which encodes a 727 amino acid protein. The initiation start codon has been tentatively identified as the first in-frame AUG of this long open reading frame. The 5' UTL of the human liver is 270 bp in length and contains 3 ATG triplets, all of which are out of frame with respect to the initiation codon of IF2_{mt}. Each of the three ATGs in the 5'-untranslated leader marks the beginning of a short upstream open reading frame. Experimental evidence indicates that uORFs strongly inhibit the translation of the downstream protein-coding sequence, presumably due to a low efficiency of ribosomal reinitiation at the downstream open reading frame. The translation of the IF2_{mt} open reading frame may be down regulated by the presence of these uORFs, but this has not been tested. The 3'-untranslated region of the cDNA is unusually short, and this 26-bp-long sequence is 85% A/Ts. Two sequences upstream from the poly (A) tail resemble the consensus sequence for polyadenylation. Therefore, one or both of them are probably used as a polyadenylation signal for the human IF-2_{mt} gene (Ma and Spremulli, 1995).

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