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Nonsense mutations in the human beta globin gene affect mRNA metabolism

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NONSENSE MUTATIONS IN THE HUMAN β GLOBIN GENE AFFECT mRNA METABOLISM

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Abstract

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A common mutation causing thalassemia in Mediterranean populations is an amber (UAG) nonsense mutation at the 39th codon of the human ß globin gene, the ß-39 mutation. Studies of mRNA metabolism in reticulocytes from patients with ß-39 thalassemia and studies using heterologous transfection systems have suggested the possibility that this mutation affects not only protein synthesis but also alters mRNA metabolism. This phenomenon has been investigated further by two approaches. A careful series of RNA expression studies were performed comparing expression of ß-39 to ß-normal (ß-nl). These experiments led to the conclusion that the defect in expression of the ß-39 mRNA resides in the nucleus. A number of nonsense and missense mutations of the ß globin gene were constructed by oligonucleotide-directed site-specific mutagenesis. Their expression was studied in a heterologous transfection system. These studies strongly suggest that the presence of a nonsense mutation (but not a missense mutation) in ß-globin mRNA decreases the accumulation of its mRNA. Decreased ß-globin mRNA accumulation can be caused by the presence of a nonsense mutation, independent of its type or location.

to my parents

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Chapter 1

Introduction to ß° thalassemia

Thalassemia is one of a group of hereditary anemias associated with defective production of the hemoglobin molecule in erythroid cells found primarily in the human population living in the Mediterranean area and in Asia. Human hemoglobin in the adult (HbA) is a tetrameric protein consisting of 2 α chains and 2 ß chains. Thalassemia is characterized by decreased, and in some cases absent, synthesis of the affected chain (α,β). The resultant aggregates of the excess chain precipitate in erythrocytes and cause their premature destruction resulting in a hemolytic anemia . Persons afflicted by severe homozygous ß thalassemia rarely live beyond the third decade and require transfusions throughout life. Elucidation of the molecular basis of the thalassemia syndromes will result in better methods for treatment, management and prevention of the disease. A complete review of the literature of hemoglobinopathies, including the thalassemia syndromes, can be found in Bunn and Forget (1986) and in Weatherall and Clegg (1982).

The study of ß thalassemia at the molecular level has formed the basis for the study of gene expression in mammalian systems. ß-thalassemia describes a condition in which there is less ß chain synthesis than normal. The genes from this disorder have been cloned and studied *in vivo* and *in vitro* through the use of techniques of recombinant DNA technology. In the normal pathway of gene expression, after the gene is transcribed, the RNA is processed and polyadenylated, transported from the nucleus into the cytoplasm, and then translated. This is diagramed in Fig. 1.1. Mutations have been described which cause thalassemia by affecting many of the steps in the process of gene expression including: 1) transcription initiation

(efficiency and specificity), 2) splicing of the mRNA precursor and 3) polyadenylation of the mRNA.

Originally, two types of ß thalassemia were recognized, $ß^+$ and $ß^\circ$, based on the amount of ß chain measureable in erythroid cells from patients peripheral blood. In $ß^+$ thalassemia, some ß chain is produced, though in reduced amounts, while in $ß^\circ$ thalassemia, there is no ß chain synthesis. It is with the causes of $ß^\circ$ thalassemia that I will be concerned.

$ß^\circ$ thalassemia can be caused both by mutations which create error in mRNA processing and by mutations which create nonsense or frameshift mutations. Except in rare cases, these mutations are associated with the complete absence of translatable mRNA (Benz et al., 1978; Old et al., 1978). In many cases, low levels of steady-state ß mRNA are associated are associated with $ß^\circ$ thalassemia (Benz et al., 1978; Old et al., 1978; Belhani et al., 1980). With the advent of gene cloning techniques, the explanation for these phenotypes can be related to alterations in structural or regulatory regions of the ß-globin locus. Many of the ß globin genes isolated from patients with $ß^\circ$-thalassemia contain single base subsitutions that create in phase translation termination or nonsense mutations. In the sequence coding for the ß globin gene there are 29 positions at which a single nucleotide substitution could produce a termination codon. In this dissertation I will concentrate on the $ß^\circ$ thalassemias caused by these substitutions.

Several nonsense mutations have been described in patients with $ß^\circ$ thalassemia. The codon at which the mutation occurs designates the name of the mutation; there are 146 codons in human ß globin mRNA. Nonsense mutations have been described at ß-15 (Kazazian et al., 1984), ß-17 (Chang and Kan,1979), ß-37 (Boehm et al., 1986), ß-39 (Moschonas et al.,

1981; Orkin et al., 1981; Trecartin et al., 1981; Pergolizzi et al., 1981; Gorski et al., 1982), ß-43 (Atweh et al., 1988) and ß -121 (Kazazian et al., 1986). Hb McKees Rocks (ß145 → term; Winslow et al., 1976) probably also has a nonsense mutation in the ß chain. Although this mutation has not been identified by direct means, the data indicate that the protein is just one amino acid short of the normal length. This mutation creates a hemoglobin variant that is not associated with thalassemia. The mutations described by DNA sequencing also vary in type of nonsense mutation. There are 3 stop codons in non-mitochondrial eukaryotic mRNAs: amber (UAG), ochre (UAA) and opal (UGA). The ß-15, ß-17, ß-39 and ß-43 are all amber (UAG) mutations. The ß-37 is an opal (UGA) mutation and the ß-121 is an ochre (UAA) mutation.

The two most thoroughly studied nonsense mutations in the human ß-globin gene are the ß-17 and ß-39 mutations. The first mutation to be described was the ß-17 (Chang and Kan, 1979) in a Chinese patient. The mutation was identified by DNA sequencing of single-stranded cDNA fragments . The gene has never been cloned. The ß-39 mutation is a common mutation which causes thalassemia in the Mediterranean population. This mutation was described simultaneously by four different groups (Moschonas et al., 1981; Orkin et al., 1981; Trecartin et al., 1981; Pergolizzi et al., 1981; Gorski et al., 1982). The DNA sequence of the cloned gene has been determined. The ß-15 mutation was described by direct DNA sequence analysis of a gene cloned from an Asian Indian patient. The ß-37 mutation, which abolishes an Ava II restriction site, was described in a Saudi Arabian patient by selective oligonucelotide hybridization of the patient's genomic DNA (Boehm et al., 1986). The ß-121mutation, which abolishes the EcoR1 site in the third exon, was described as a spontaneous mutation in a child born to a Greek-Cypriot mother and a Polish father. The ß-121 gene was cloned and the mutation identified by direct DNA sequencing. The ß-43 was

recently described in a Chinese patient by direct DNA sequencing of the cloned ß-globin gene (Atweh et al., 1988). Further study of the original patient with the ß-17 mutation has shown that this patient is heterozygous for the ß-43 mutation (George Atweh, personal communication). This handful of mutant ß globin genes are those in which a single base substitution creates a nonsense mutation at the site of the mutation.

Studies of mRNA metabolism of the ß-17 and ß-39 mRNAs in patients' peripheral blood have suggested that nonsense mutations have a novel effect on mRNA metabolism. Translatability of the mRNA appears to affect mRNA metabolism. Chang et al. (1979) examined the level of ß-17 mRNA present in one patient's reticulocytes and found that it was 15% of normal. Two groups (Trecartin et al., 1981; Moschonas et al., 1981) examined the level of ß-39 mRNA in reticulocytes from two different patients with ß° thalassemia and found it to be 3-5% of normal. For both the ß-17 and ß-39, the only mutation present in each is the mutation that creates the premature termination codon. Takeshita et al. (1984) confirmed that the levels of ß-39 mRNA are reduced *in vivo* but extended the finding with a kinetic analysis of ß-globin synthesis and stability. Patients' erythroblasts were incubated for 20 minutes with ^{3}H-uridine (the "pulse") and "chased" with nonradioactive uridine for 20 hours. The ß/α ^{3}H–mRNA ratio was measured at the end of the pulse and the end of the chase in the nucleus and in the cytoplasm. It was found that the small amount of ß mRNA in the cytoplasm was stable for 20 hours. However, the level of mRNA during the pulse alone was higher in the nucleus than in the cytoplasm by 2 to 2.5-fold. These results suggest that the defect in ß-39 mRNA accumulation occurs first in the nucleus shortly after or concurrent with transcription or during nuclear-cytoplasmic transport of the mRNA.

It is more convenient to study mRNA metabolism in an *in vitro* system than it is to study it in erythroblasts from patients' peripheral blood. Fortunately, the results obtained from *in vivo* studies were reproducible in a heterologous transfection system by surrogate or reverse genetics (Takeshita et al., 1984; Nienhuis et al., 1984). The cloned ß-39 and normal ß globin genes (ß-nl) were expressed from their own promoters on a vector, PTL9, that contains both the SV40 origin of replication and the SV40 enhancer. Takeshita et al. (1984) expressed their constructs in COS cells, a monkey kidney cell line that constituitively expresses the SV40 T antigen while Nienhuis et al. (1984) expressed their constructs in HeLa cells. In both cases the results are similar. The level of ß-39 mRNA is reduced five to ten-fold when compared to ß-nl mRNA when total mRNA is analyzed by S1 nuclease analysis. The level of ß-39 mRNA is also reduced when the mRNA is analyzed in nuclear and cytoplasmic fractions. To examine whether cytoplasmic instability was the cause for decreased level of ß-39 mRNA, the transfected cells were incubated for 3-12 hours with actinomycin D. The mRNA levels for both the ß-nl and ß-39 mRNAs were essentially the same before and after actinomycin D incubation. These results argue that cytoplasmic stability is not the cause of the defect in ß-39 mRNA metabolism. The 3-12 hour period of actinomycin D incubation should be sufficient to detect a defect in ß-39 mRNA stability since it has been shown that the reduction in ß-39 steady-state levels occurs within 20 minutes of transcription *in vivo* (Benz et al., 1981). These results suggest that the defect in metabolism of the ß-39 mRNA is intranuclear, either in nuclear stability or in nuclear-cytoplasmic transport.

Atweh et al.(1988) have used a heterologous transfection system to study mRNA metabolism of the ß-43 (UAG) mRNA. They used a different expression vector than the one used above, a vector known as πSVplac. This vector also contains the SV40 origin of replication and the enhancer region. The constructs were transfected into HeLa cells.

Steady-state ß-43 mRNA was reduced when compared to ß-nl in the heterologous system, mimicking the *in vivo* observations. The fidelity of these studies is neither vector nor cell type dependent.

In contrast to the results obtained by several groups, Moschonas et al. (1981) found no difference in steady-state mRNA levels for the ß-39 and ß-nl mRNAs after expression in HeLa cells. They studied expression of ß-39 mRNA from a genomic fragment identical to the one subcloned by Takeshita et al. (1984) and Humphries et al. (1984), the 4.7 kb Bgl II fragment. The expression vector they used, an SV40-pBR328 construct, was different from the two described above. The reason for the difference between these results and those described in the experiments above is not known.

Y.W. Kan and his colleagues have proposed a potential gene therapy for ß-thalassemia caused by nonsense mutations by introduction of the appropriate suppressor tRNA into bone marrow cells. Although this goal has yet to be achieved, important steps toward this goal have been accomplished. Chang et al. (1979) were the first to suppress a nonsense mutation in human mRNA. They translated poly-A^+ ß-17 mRNA isolated from patient's peripheral blood in a rabbit reticulocyte lysate system in the presence of a serine-inserting amber suppressor tRNA from *Saccharomyces cerevisiae* . The expected sized ß-globin chain was obtained. Temple et al. (1982) extended these studies in a cellular system. They studied suppression of the ß-17 mutation in *Xenopus* oocytes. *Xenopus* oocytes were microinjected with a synthetic human suppressor $tRNA^{lys}$ gene. Twenty hours later, poly A^+ ß-17 mRNA was microinjected into these cells. After labeling of the cellular protein with ^{35}S-methionine, the presence of the ß- chains was examined by immunoprecipitation. The presence of the suppressor tRNA allowed the synthesis of the authentic ß chain. It has been much more

difficult to successfully express the suppressor tRNA constitutively in tissue culture cells (Ho et al., 1986; Hudziak et al., 1982), and because of this investigators are now designing systems with inducible expression of suppressor tRNAs. There are at least three conditions which must be met in order to obtain efficient suppression in mammalian cells: 1) high levels of expression of suppressor tRNA are necessary, 2) the context of the nonsense mutation must be favorable for suppression and 3) amino acylation of the suppressor tRNA must occur.

Takeshita et al. (1984) used nonsense suppression as a tool to investigate the mechanism of the reduction in ß-39 mRNA levels. They co-transfected a *Xenopus* tyrosine-inserting suppressor tRNA with the ß-39 and ß-nl genes. In the presence of the suppressor tRNA, the level of ß-39 mRNA was restored to normal; there was no effect on the level of ß-nl mRNA. These results suggest that it is the presence of the translation termination codon that causes the defect in mRNA metabolism. Nonsense suppression will be discussed in detail in chapter 3.

There are seven frameshift mutations which cause ß° thalassemia (Bunn and Forget, 1986). These are small deletions or insertions that change the translational reading frame of the ß-globin gene and place a premature termination translation termination codon in the new reading frame. Of these mutated ß-globin genes, only two have been analyzed for defects in mRNA metabolism. The first gene studied was cloned from a Turkish individual (Orkin and Goff, 1981). DNA sequence analysis indicates that the gene contains a dinucleotide deletion at codon 8 that produces a termination codon at the position of the new 21st codon. Although the data are not shown, the authors state that the mutant ß mRNA is deficient in this patient's reticulocytes. The second gene studied was cloned from a Kurdish Jewish individual (Kinniburgh et al., 1982). Analysis of the cloned gene indicates that it contains a 1 base pair deletion at codon 44 that produces a termination codon at the new 60th codon. The level of

ß-44 (-1) mRNA in patients' reticulocytes is reduced about 40 fold when compared to normal. Further studies using actinomycin D indicate that the half-life of the ß-44 (-1) is reduced from the normal 24 hour half-life to 30 minutes (Maquat et al., 1981). However, they did not fractionate the mRNA and therefore may be observing an intranuclear phenomenon. This remains the only ß-globin gene with a nonsense mutation for which there is presented evidence for cytoplasmic instability.

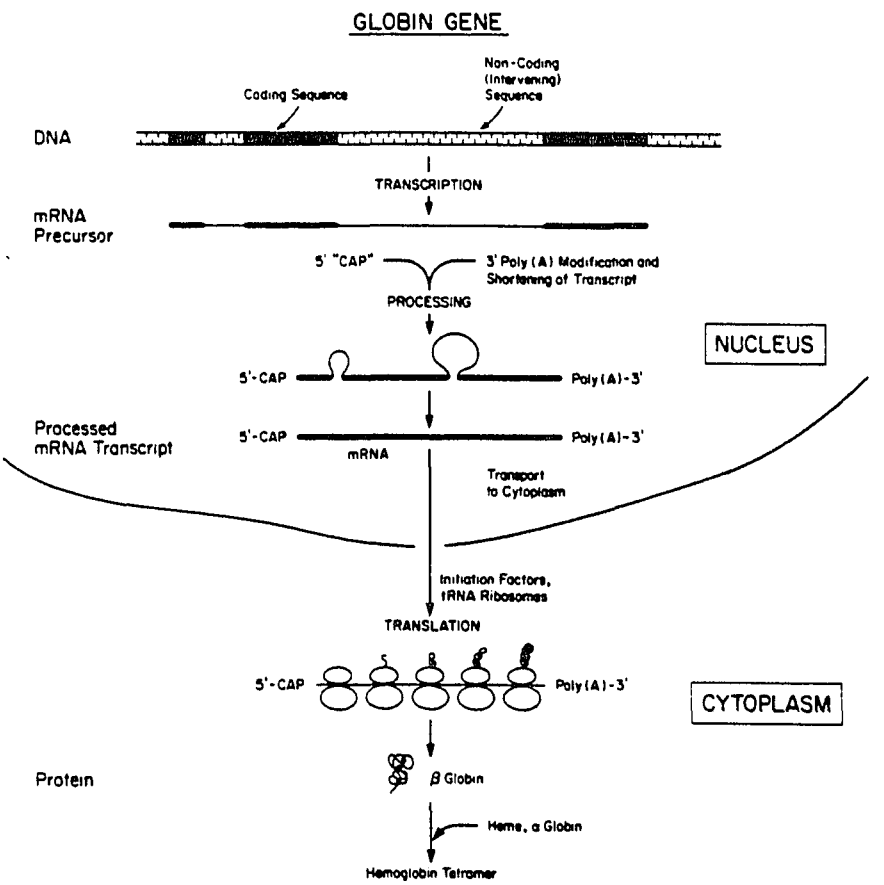
Maquat and Kinniburgh (1985) were not able to reproduce these findings in a heterologous transfection system. They found that the ß-44 (-1) mRNA in HeLa cells was relatively stable when compared to normal. They conclude that there are factors in erythroid cells but not in cervical carcinoma cells that modulate the instability of the ß-44 (-1) mRNA. Unfortunately, they did not include in their studies a mutated ß- gene which is known to produce a decrease in mRNA levels like the ß-39. Because of this, it is difficult to say whether their observations for the ß-44 (-1) are a property of this mRNA or merely an artifact of this system.

In addition to these ß thalassemia lesions, there are two α thalassemia lesions which are associated with a decrease in the level of their mRNA. A mutation in the α2–globin gene which changes the translation initiation codon ATG to ACG creates a nonfunctional mRNA (Pirastu et al., 1984). The α2:α1 ratio was reduced from the normal of 3:1 to 1:1. The steady-state mRNA levels of the mutant α2-globin gene were reduced by about 70%. Reduced mRNA levels have also been reported for hemoglobin Constant Spring (HbCS). HbCS has an elongated α chain of length 172 amino acids instead of the normal 141. The elongated α chain is thought to result from a mutation in the normal translation termination codon which allows

read-through of the 3' untranslated region until another in phase termination codon is reached. The amount of HbCS mRNA present in patients' peripheral blood is minimal. The mechanism for the reduced mRNA levels for either of these α thalassemia lesions is not understood.

For nonsense and frameshift mutations in ß-globin genes, there is an effect of translatability of the mRNA on mRNA metabolism. Furthermore, as seen with the ß-39 mutation, translatability can affect intranuclear mRNA metabolism. This would be a new level of control in the pathway of gene expression. I chose to characterize this phenomenon in two ways. My first approach was to investigate the mechanism of the defect in intranuclear metabolism of the ß-39 mRNA. My second approach was to extend the findings for the ß-39 mRNA to other nonsense mutations in the human ß globin gene. In this way I have undertaken steps toward understanding the normal and abnormal processes of mRNA metabolism.

Fig.1.1. An outline of the pathway of ß-globin gene expression. A precursor mRNA is transcribed from the DNA in the nucleus. The precursor mRNA is capped, polyadenylated and spliced before it is transported out of the nucleus into the cytoplasm. In the cytoplasm the mRNA is translated into protein.



Chapter 2

Studies on the mechanism of the metabolism of the human ß-globin mRNA

Introduction

Many of the steps in ß-globin gene expression have been well-studied. mRNA is transcribed from DNA, spliced, polyadenylated, and then translated into protein in the cytoplasm. Less is known about the determinants of nuclear and cytoplasmic globin mRNA stability and about nuclear-cytoplasmic transport. The ß-39 gene causes thalassemia because it encodes a truncated non-functional globin protein chain. Although the nonsense mutation at codon 39 affects translation, studies from our laboratory and others suggest that this mutation has an additional effect on mRNA metabolism (Takeshita et al., 1984; Humphries et al., 1984). There is reduced accumulation of the ß-39 mRNA when compared to ß-nl in both patient's red blood cells and in a heterologous transfection system. Since there is also reduced accumulation of ß-39 mRNA in the nucleus, it has been hypothesized that the defect in expression of the ß-39 gene is intranuclear.

The unusual phenotype of the ß-39 mutation suggests that a novel element in the regulation of gene expression may be uncovered by further investigation. Cell lines which bear the ß-39 and ß-nl genes integrated into their genome were constructed and analyzed. I have investigated potential differences between expression of the ß-39 and ß-nl genes in 1) steady-state levels of total, nuclear and cytoplasmic mRNA, 2) mRNA half-life, 3) accuracy of mRNA initiation and splicing, 4) mRNA polyadenyation and 5) transcription initiation. I have taken a novel approach by studying mRNA half-life using a biologic assay. AF8 cells, a BHK-derivative that are temperature-sensitive for RNA polymerase II, were transfected with the ß-nl and ß-39

genes. At the non-permissive temperature, RNA polymerase II transcription is blocked. At present there is no system in which nuclear-cytoplasmic transport can be directly studied. In addition, the effect of translation inhibitors was tested on ß-nl mRNA metabolism. In this chapter I will present and discuss the experiments that I have designed to determine at which step in gene expression the defect in ß-39 mRNA metabolism occurs.

Materials and Methods

Cell lines. AF8 cells were obtained from Renato Baserga, Temple University. AF8 cells are a temperature-sensitive RNA polymerase II derivative of BHK, a Syrian hamster cell line (Meiss and Basilico, 1972; Burstin et al., 1974). They were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum, 2 mM glutamine, 50 U/ml of penicillin, 50 µg/ml of streptomycin. The cells were grown at the permissive temperature of 34°C. Experiments at the non-permissive temperature were performed at 40°C.

Bacterial strains. HB101 was used for routine growth of plasmid DNA. The genotype of HB101 is *F-, hsdS 20 ($r-_B$, $m-_B$), recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Smr), xyl-5, mtl-1, supE44, λ–.*

Plasmids. The ß-39 and ß-nl globin genes used in these experiments were subcloned into an SV40-based expression vector by Kenichi Takeshita (Takeshita et al., 1984). The ß-39 gene was cloned from a patient with ß°-thalassemia and the 5 kilobase (kb) BglII fragment was subcloned into a pTL9 expression vector. PTL9 (Humphries et al., 1982.) consists of the SV40 72 base pair (bp) repeats, an SV40 origin of replication and pBR322 sequences. The ß-nl gene was subcloned into this vector by exchanging the 1.8 kb BamH1 fragment of the ß-nl gene for the ß-39 gene. The human ß-nl gene was obtained from Tom Maniatis (Lawn et al., 1978). When I obtained the ß-39 and ß-nl genes subcloned into the pTL9 expression vector, I believed that the vector was as described in Humphries et al., 1982. By restriction site analysis I was able to confirm that the vector is actually an earlier generation than that described, and

contains the "poison" sequences described by Lusky and Botchan (1981).

PSV2neo (Southern and Berg, 1982) was obtained from Michael Liskay, Yale University.

γ actin (Gunning et al., 1983) was obtained from Larry Kedes, Stanford University.

PSV2G, a plasmid bearing the early and late genes of Sv40, was obtained from Renato Baserga, Temple University.

Plasmids were grown and harvested by the alkaline lysis procdure (Maniatis et al., 1982). All plasmid preparations of the ß-39 were checked for the presence of the mutation by digestion with Mae I.

Transfections. Transfections were performed by the calcium phosphate method (Graham and van der Eb, 1973; Wigler et al. 1979). Five µg of either the ß-nl or ß-39 plasmid DNA was mixed with 0.5 mg of pSV2neo and 12 µg of calf thymus DNA in 0.5 ml 2X HEBS (280 mM NaCl, 50 mM Hepes, 1.5 mM sodium phosphate, pH 7.05). An equal volume of 0.25 M $CaCl_2$ was added, the solution was vortexed and incubated at room temperature for 20-30 minutes. The precipitate was added to sub-confluent AF8 cells in 100 mm plates for 6-12 hours. DMSO shock (20%) was performed, the cells were washed with PBS, and incubated overnight at 37°C. The next day the cells were sub-cultured and portions (1 ml, 0.8 ml, 0.4 ml and 0.2 ml) of a 4 ml cell suspension were plated. On the following day the medium was changed and G418 (1mg/ml) was added for 7 days. On the eighth day G418 concentration was reduced to 0.5 mg/ml and continued for 7 more days. At this time, individual colonies of each type were picked with cloning rings and expanded. One plate of 15

colonies of the ß-nl transfection was pooled. All cell lines were maintained in G418 selection (0.5 mg/ml). The cell lines were frozen in DMSO and stored in liquid nitrogen.

DNA isolation. Genomic DNA was isolated from each cell line according to the method of Liskay and Evans, 1980.

RNA isolation. All RNA was isolated from sub-confluent T-150 cm^2 flasks. Total RNA was isolated by the differential precipitation method of Chirgwin et al (1979). Cytoplasmic and nuclear mRNA were isolated by NP40 lysis using vanadyl ribonucleoside as an RNAse inhibitor (Favaloro et al., 1980). Nuclear RNA was isolated from the pellet using the method of Chirgwin et al (1979).

Southern blots. Southern blots (Southern,1975) were performed as described by Maniatis et al.(1982).

Northern blots. Northern blots were performed as described by Maniatis et al. (1982).

Nick Translations. Nick-translated ^{32}P-labelled probes for Southern and Northern blots were prepared using the BRL nick translation kit, according to the manufacturers' directions. All probes were prepared as agarose-gel purified fragments by standard procdures (Maniatis et al., 1982). Probes: 1.8 kb BamH1 fragment of the human ß-globin gene, Xba-Pst fragment of γ actin, Kpn-R1 fragment of pSV2G.

Half-life experiments. Two kinds of experiments were performed to determine the half-life of the ß-39 and ß-nl mRNA's. One experiment took advantage of the ts RNA

polymerase II in AF8 cells. Cells bearing either the ß-nl and ß-39 genes were placed at the non-permissive temperature (40°C) for up to 50 hours. At this temperature, RNA polymerase II activity declines with a half-life of 12 hours. Total RNA was isolated at various time points and analyzed by Northern blots. Relative half-life of the ß-nl and ß-39 genes was also determined by incubation of the cell lines with actinomycin D (10 µg/ml) for up to 3 hours. At this time, RNA was isolated and analyzed by Northern blots.

S1 nuclease analysis. S1 nuclease analysis of total RNA was performed according to the method of Ley et al. (1982), using a uniformly labelled M13 probe.

Purification of poly A+ mRNA. Poly A+ mRNA was prepared from total RNA or from cytoplasmic RNA by passsage over oligo-dT cellulose according to the method of Aviv and Leder (1972). RNA was passed twice over this column.

Nuclear run-offs. Nuclear run–offs were performed according to Engel et al.(1985). Incorporation of ^{32}P-UTP was standardized to be linear for a reaction length of 10-20 minutes and 3 X 10^7 nuclei. Approximately 2 X 10^6cpm were hybridized to Southern blots of plasmid DNA. The two plasmids used on the Southern blot contained subclones of the human ß-globin gene and γ actin.

Translation inhibitors. Cycloheximide (25 µg/ml), an inhibitor of peptide translocation, was added to AF8 cells for 3 hours. This concentration of cycloheximide has been shown to inhibit 90-95% of protein synthesis. Concentrations of cycloheximide 10 µg-500 µg/ml were found to arrest protein synthesis in AF8 cells at 1 hour after addition (Sergio Ferrari and Renato Baserga, personal communication). Sodium fluoride (NaF, 10

mM), an inhibitor of translation initiation, was added to AF8 cells for 3 hours. After 3 hour pulses, total RNA was isolated and analyzed by Northern blots.

Results

Transfection of AF8 Cells. Stable transformants of AF8 cells were obtained by co-transfection of either the ß-39 gene or the ß-nl gene with pSV2neo (Fig. 2.1) The ß-globin genes were cloned into a pTL9 expression vector. Colonies resistant to G418 (0.5 mg/ml) after 2 weeks of selection were cloned and expanded. Cells from 4 colonies bearing the ß-nl gene, including one which is a pool of 15 colonies, and cells from 4 colonies bearing the ß-39 gene were studied in detail.

Southern blot analysis. Southern blot analysis of genomic DNA from these cell lines showed an intact ß-globin gene in all cases (data not shown). In some cells lines a small amount of rearrangement was also seen. In the ß-nl cell lines the copy number varied from 2 to 10 copies/cell while in the ß-39 cell lines the copy number was always greater than that of the ß-nl cell lines and varied from 10-50 copies/cell.

Northern blot analysis. Total RNA was isolated from subconfluent culutres of ß-nl and ß-39 cell lines. Four ß-nl and four ß-39 cell lines, as well as the AF8 parent cell line, were analyzed. Northern blot analysis of 10 µg of each was performed with a nick-translated ß globin-specific probe. Two µg of reticulocyte mRNA was run to check for the correct size ß-globin transcript. As shown in Figure 2.2, in all cases the amount of 10S globin mRNA was greatly reduced in the ß-39 cell lines when compared to the ß-nl cell lines. Even if cell lines of similar copy number are compared (wt.3 vs. 39.1), the amount of 10S mRNA is reduced about 5-fold in the ß-39 line when compared to the ß-nl cell line. The pooled line is designated wt.0. There are also a number of high molecular weight transcripts present in the ß-39 cell lines which hybridize to the ß-globin probe. At first I was intrigued by these bands since it was

possible that they represented unspliced pre-ß-39 mRNA transcripts. I performed Northern blots using a ß-globin intron-specific fragment (the Hinf fragment from the ß-globin large intron) and saw no difference in hybridization between the ß-nl and the ß-39 cell lines (data not shown). I than performed Northern blots using SV40-specific sequences (pSV2G) as a probe and saw hybridization in both the ß-nl and ß-39 lanes (data not shown), although no discrete bands were present. It is likely that the high molecular weight transcripts represent read-through transcripts. The presence of these transcripts is probably related to copy-number, since they are also present in the ß-nl cell line with the highest copy number, wt.3.

To show that the same amount of RNA was loaded in each lane, the nitrocellulose was stripped and re-hybridized to a nick-translated human γ actin probe. In Figure 2.2, the bottom panel depicting the γ actin hybridization indicates that slightly less RNA was loaded in some of the ß-nl lanes.

In this experiment I have shown that the level of ß-39 mRNA is reduced when compared to ß-nl in a stable expression system.

Half-life experiments. To analyze the relative half-life of ß-nl (wt.1) and ß-39 (39.2) mRNA's in AF8 cell lines, cells were placed at the non-permissive temperature (40°) for 30 and 50 hours. At this temperature, RNA polymerase II activity decays with a half-life of about 10 hours and cannot be measured by 48 hours (Rossini et al., 1980). Therefore, at 40°C, synthesis of new mRNA's ceases. As a control, total RNA was isolated from AF8 cells before shift-up to the non-permissive temperature. Ten µg of each RNA was analyzed on a Northern blot using a ß-globin specific probe (Fig. 2.3). The data show that both the ß-nl and the ß-39

10S mRNA's are stable up to 50 hours after shift-up to the non-permissive temperature. To check for even loading of mRNA, following hybridization the nitrocellulose was stained with methylene blue which stains the rRNAs.

To confirm the biological half-life experiments, an actinomycin D incubation was performed. Actinomycin D was added to subconfluent AF8 cells bearing either the ß-nl (wt.1) or ß-39 (39.2) genes for 3 hours. Total mRNA was isolated before addition of the drug and at 3 hours after the addition of the drug. Equal amounts (10 µg) were analyzed on a Northern blot. (Fig. 2.4) The blot indicates that, at least for 3 hours, both the ß-nl and the ß-39 mRNA's are stable in cell lines.

Splicing and initiation of ß-39 mRNA. S1 nuclease analysis was performed on total RNA from the ß-nl and ß-39 cell lines. A uniformly labelled M13 probe was used. This probe hybridizes to all 3 exons of the human ß-globin gene. With this probe I could examine whether the ß-39 mRNA was incorrectly spliced or initiated. As shown in Figure 2.5, both the ß-nl and ß-39 mRNA's are correctly initiated and correctly spliced.

Polyadenylation of ß-39 mRNA. Polyadenylated mRNA was isolated from 1 ß-nl (wt.1) and 1 ß-39 (39.4) cell line by passage over oligo-dT cellulose. The polyadenlylated fraction was collected and concentrated by ethanol precipitation. Analysis of 5 µg each of total RNA, poly-A- mRNA and poly-A+ mRNA on a Northern blot with a ß-globin specific probe shows enrichment of the 10S mRNA in the poly-A+ fraction (Fig. 2.6). No hybridization is seen to the poly-A- lane in either case. Both the ß-nl and ß-39 mRNA's are polyadenylated.

Fractionation of ß-39 mRNA. Nuclear and cytoplasmic mRNA were fractionated

from 1 ß-nl cell line (wt.1) and from 1 ß-39 cell line (39.2). The relative amount of ß-nl and ß-39 mRNA present in each was investigated. The results of a Northern blot demonstrate that the relative amounts of each ß mRNA are the same whether total or cytoplasmic mRNA was isolated (data not shown). Figure 2.7 shows a Northern blot of equal amounts of poly-A+ total mRNA and poly-A+ cytoplasmic mRNA for the ß-nl and the ß-39 cell lines. It shows that cytoplasmic poly-A+ mRNA from 39.2 accumulates in reduced amounts when compared to wt.1. Nuclear ß-39.2 mRNA is also present in reduced amounts when compared to ß-nl (data not shown).

Transcription initiation rate of ß-39 mRNA. Nuclear run-off transcription was performed on 1 ß-nl (wt.1) and 1 ß-39 (39.2) cell line. The rates of transcription initiation of the 2 globin mRNA's were compared (Fig 2.8). The rate of transcription initiation of each ß mRNA was also compared to the rate of transcription initiation of other plasmid sequences in the transfected construct. Each lane was traced by densitometry and the amount of hybridization to the ß-globin sequences was normalized to the amount of hybridization to the plasmid sequences (data not shown). When these calculations were made, the 39.2 mRNA was found to be initiated at a rate about half that of the wt.1 mRNA. This difference is not large enough to account for the difference in steady-state levels of the ß-nl and ß-39 mRNAs.

Translation inhibitors. Cycloheximide (25 µg/ml) or sodium fluoride (10 mM) were added to 1 ß-nl cell line to investigate whether a reduction in the level of ß-nl mRNA could be achieved by the presence of translation inhibitors. Total RNA was harvested after 3 hours of incubation. The relative amount in each was compared by Northern blot analysis. Fig 2.9 shows that there is no change in the relative amount of ß-globin mRNA or γ actin for either the cycloheximide or sodium fluoride treated cells.

Fig.2.1. Transfection of AF8 cells. Shown is the outline of the procedure used to produce AF8 cell lines with stably integrated copies of either the ß-nl or ß-39 genes. The ß-nl or ß-39 globin genes were sub-cloned into an SV40 expression vector and co-transfected with a pSV2neo plasmid into AF8 cells. AF8 cells are a temperature-sensitive RNA polymerase II derivative of BHK cells and do not express ß-globin in the untransfected state. After selection in G-418, individual neomycin resistant colonies and pools of neomycin resistant colonies (see text) were expanded and analyzed for globin gene expression.

STABLE EXPRESSION SYSTEM

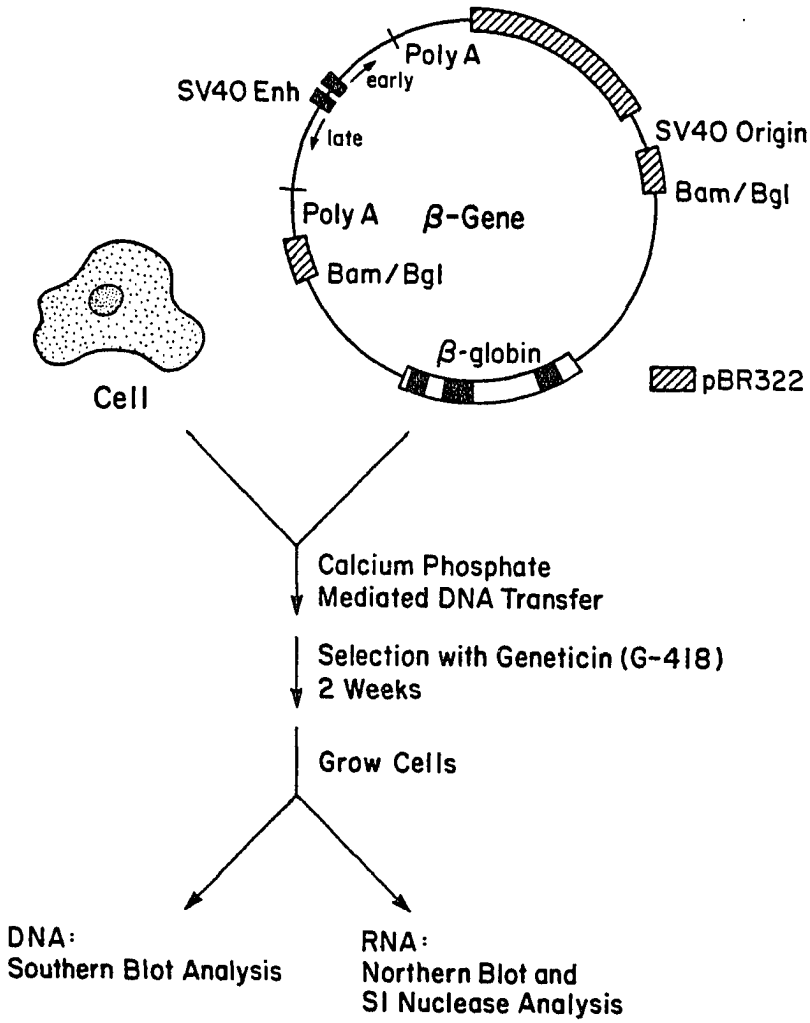


Fig.2.2. Steady-state mRNA analysis of ß-nl and ß-39 AF8 cell lines.

Shown is a Northern blot of 10 μg of total mRNA from 4 ß-39 AF8 cells lines and from 4 ß-nl cell lines. The ßwt.0 cell line is made form a pool of neomycin resistant colonies. All the other cell lines are from single neomycin resistant colonies. The top panel shows hybridization to a ß-globin probe; the normal-sized ß-globin mRNA is indicated. The larger hybridizing transcripts are discussed in **Results-Northern blot analysis.** The bottom panel shows the rehybridization of a γ actin probe to the same filter.

Reduced Accumulation of β-39 Thalassemic mRNA in Stable AF8 Cell Lines

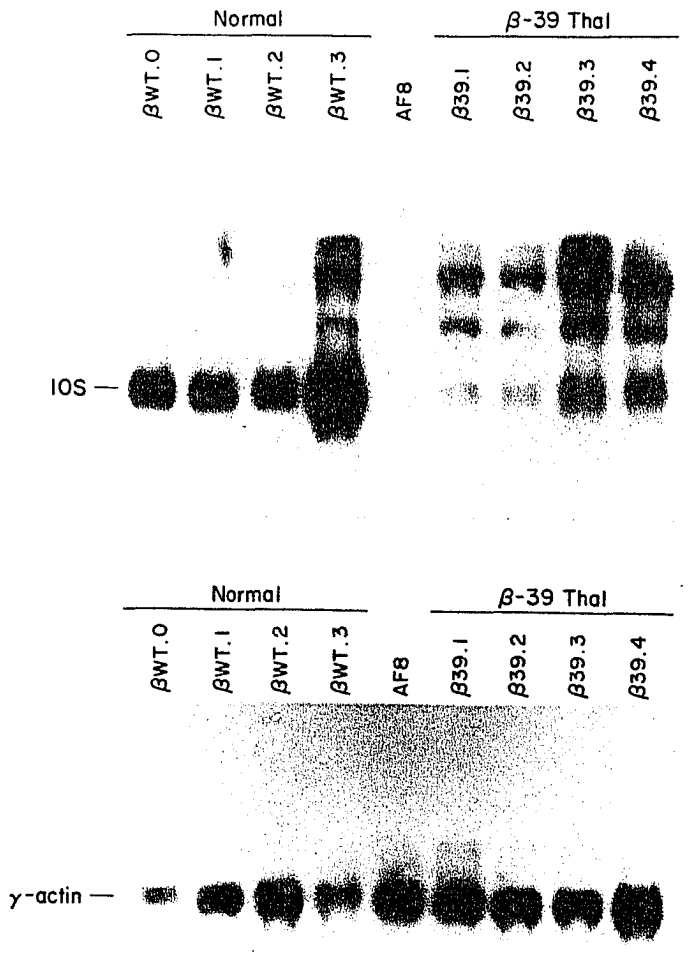


Fig.2.3. Biological half-life analysis of representative ß-nl (wt.1) and ß-39 (39.2) AF8 cell lines. Shown is a Northern blot using a ß-globin specific probe of 10 µg of total RNA from both ß-nl and ß-39 cell ines at 2 time points after shift-up to the non-permissive temperature.

Relative Stability of Normal
and β-39 Thalassemic mRNA's in AF8 Cells

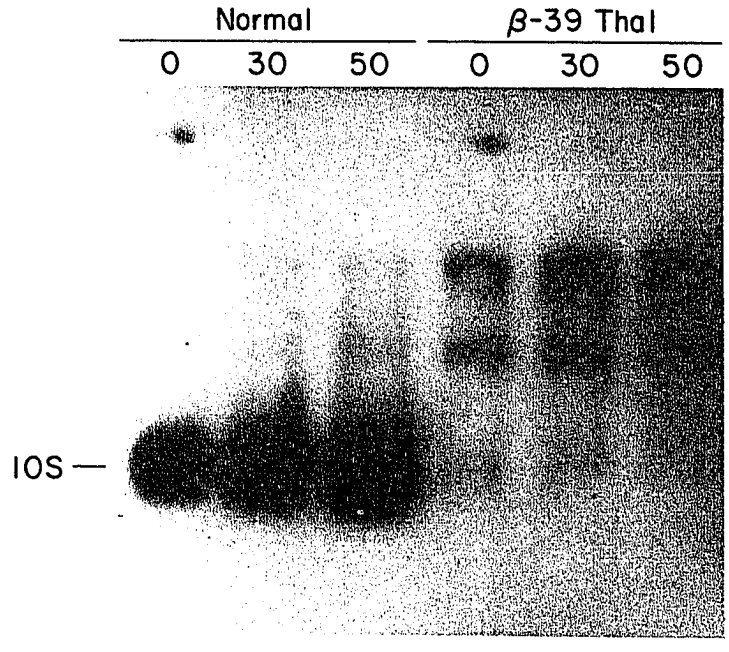


Fig.2.4. Half-life analysis of representative ß-nl and ß-39 AF8 cell lines by actinomycin D. Representative ß-nl (wt.1) and ß-39 (39.2) AF8 cell lines were used in this analysis. Shown is a Northern blot using a ß-globin specific probe on 10 µg of total RNA before and 3 hours after addition of actinomycin D.

RELATIVE STABILITY OF NORMAL AND β-39 THALASSEMIC mRNA's IN AF8 CELLS

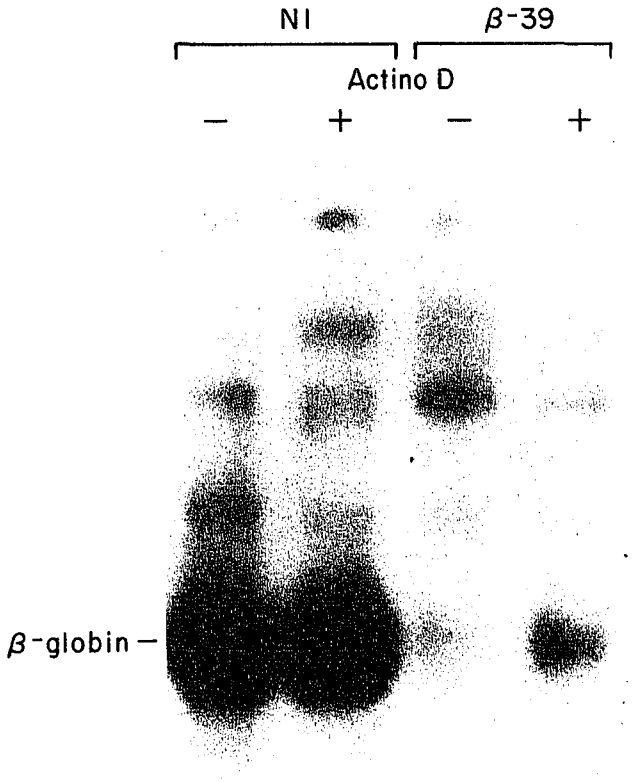


Fig.2.5 Splicing and intiation of ß-nl and ß-39 mRNA. Shown is an S1 nuclease analysis using a uniformly labelled M13 probe of both the ß-nl and ß-39 AF8 cell lines. The individual exons are indicated. The double band seen in all lanes at the exon 1 position is an S1 artifact. The ß-39 panel was exposed 7 timess longer than the ß-nl panel.

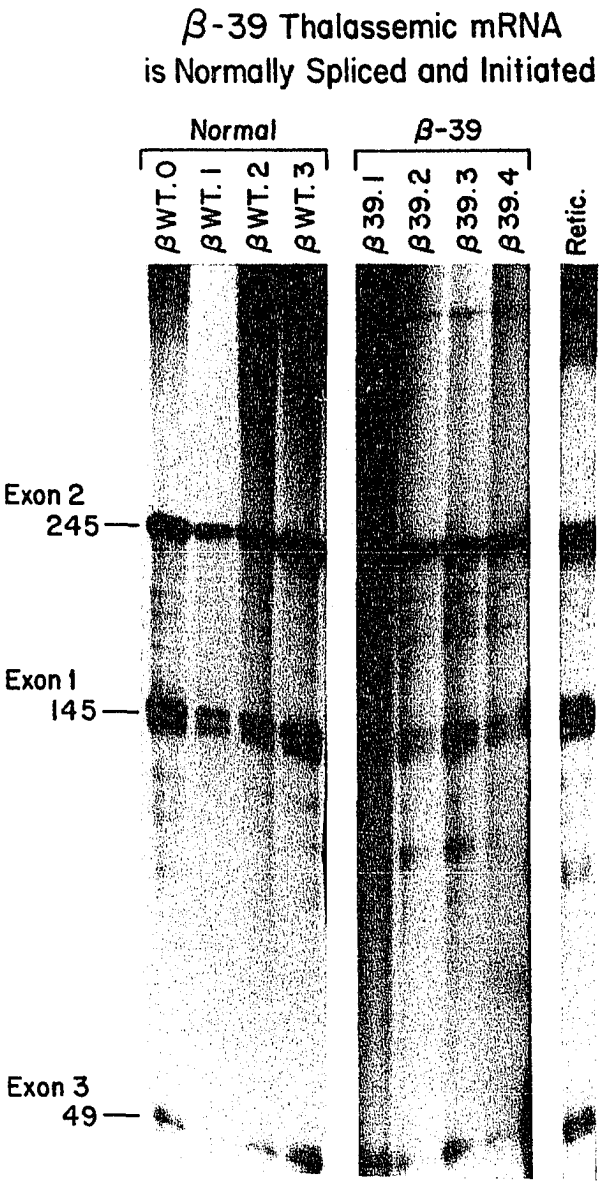


Fig.2.6 Polyadenylation of ß-39 mRNA. Shown is a Northern blot using a ß-globin specific probe of 5 µg of total RNA, poly A^- and poly A^+ RNA from 1 ß-nl (wt.1) and 1 ß-39 (39.4) cell line. The poly A^+ RNA was isolated by two passages over oligo-dT cellulose.

Polyadenylation of β-39 Thalassemic mRNA in AF8 Cells

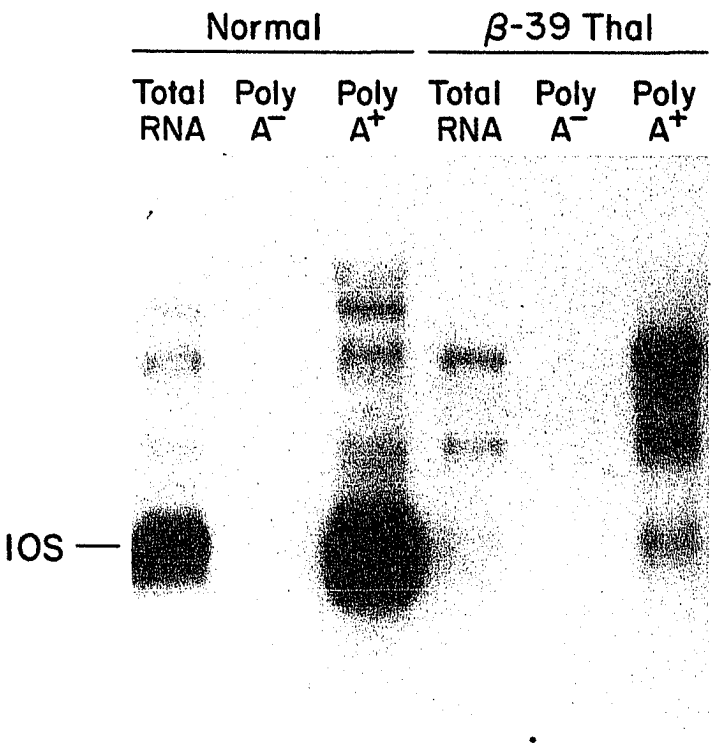


Fig.2.7. Fractionation of ß-39 mRNA. Shown is a Northern blot using a ß-globin specific probe of 5 µg ß-nl (wt.1) or ß-39 (39.2) poly A^+ RNA isolated from either the whole cell or form the cytoplasm of the AF8 cell lines.

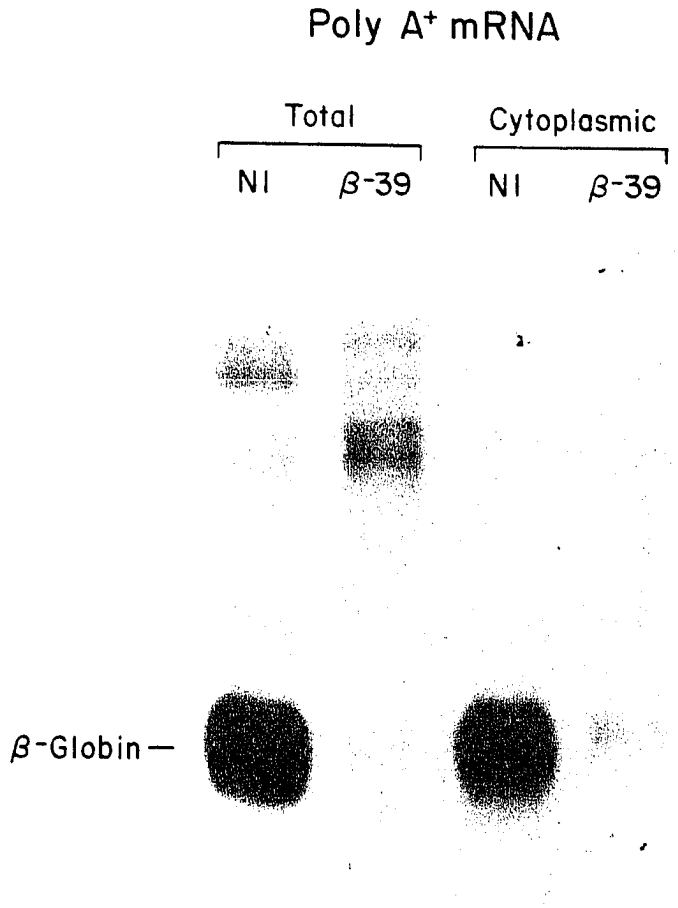


Fig.2.8. Transcription initiation rate of ß-39 mRNA. ^{32}P-labelled nuclear run-off mRNA from 1 AF8 ß-nl cell line (wt.1; top panel) and from 1 AF8 ß-39 cell line (39.2; bottom panel) was hybridized to plasmid, ß-globin and γ actin DNA sequences immobilized on a nitrocellulose filter.

NUCLEAR RUN-OFF ANALYSIS OF NORMAL AND β-39 mRNA's IN AF8 CELLS

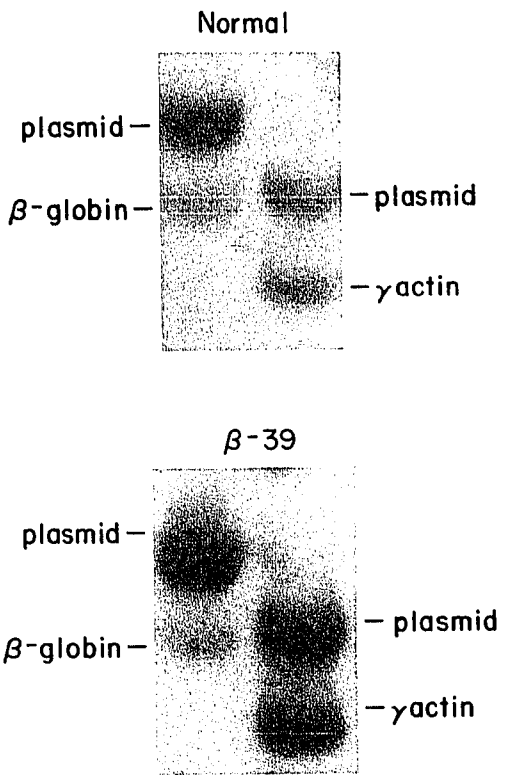
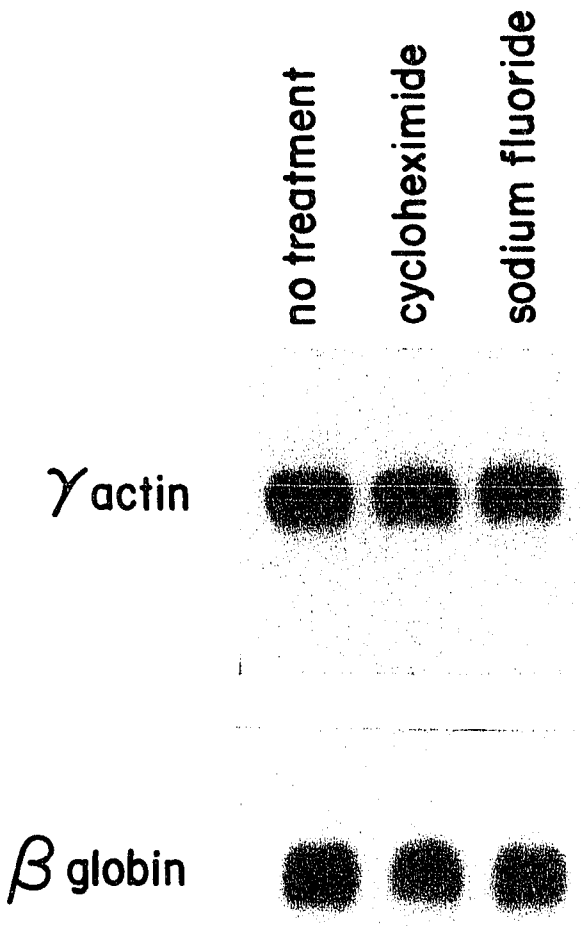


Fig.2.9. The effect of translation inhibitors on ß-globin mRNA levels.

Shown is a Northern blot of 10 μg of total mRNA from a ß-nl (wt.1) cell line before and after 3 hours of treatment with either cycloheximide or sodium fluoride. The top panel shows hybridization to a γ actin probe and the bottom panel to a ß-globin probe.



Discussion

Summary. The aim of these experiments was to investigate the mechanism which reduces accumulation of the human ß-39 mRNA when compared to ß-nl in mammalian cells. It had been suggested from studies in transient expression systems that the defect in expression of the ß-39 mRNA was likely to be intranuclear (Takeshita et al, 1984, Humphries et al., 1984). The results presented here have confirmed and extended their work. In order to have a constant source of mRNA, AF8 cell lines which constitutively express either the ß-39 mRNA or the ß-nl mRNA were constructed. The phenomenon of decreased ß-39 mRNA accumulation when compared to ß-nl was reproduced in these stable cell lines for total, cytoplasmic and nuclear ß mRNA. I have applied the techniques of molecular biology to study these well-characterized cell lines in order to focus on the point in the pathway for gene expression at which the defect in expression occurs. I investigated whether there was a defect in 1) the transcription initiation rate, 2) the accuracy of transcription initiation and mRNA splicing, 3) polyadenylation or 4) the mRNA half-life. I found that 1) transcription initiation frequencies of the ß-39 and the ß-nl mRNA's are approximately equal, 2) ß-39 and ß-nl mRNA's are correctly initiated, 3) ß-39 and ß-nl mRNA's are correctly spliced, 4) ß-39 and ß-nl mRNA's are polyadenylated, 4) ß-39 and ß-nl mRNA's are stable for up to 50 hours after shift-up to the non-permissive temperature in AF8 cells, and 5) ß-39 and ß-nl mRNA's are stable for at least 3 hours in actinomycin D. I have investigated and eliminated a number of possible explanations for the defect in expression of the ß-39 mRNA . However, I was not able to investigate 2 remaining control points for gene expression: intranuclear mRNA stability and nuclear-cytoplasmic mRNA transport. Based on the data that I have generated, the hypothesis that I favor to explain the reduced accumulation of the ß-39 mRNA is as follows. Either there is a primary defect in nuclear-cytoplasmic transport of the ß-39 mRNA with a

secondary defect in intranuclear stability or there is a primary defect in intranuclear stability of the ß-39 mRNA.

Experimental design. Although the techniques I have used are fairly standard, there are a few inherent problems with them that I would like to discuss. As I mentioned in the **Results** section, there are a number of high molecular transcripts present in the ß-39 cell lines which hybridize to the ß-globin probe (see Fig.2.2). Because they also hybridize to an SV40-specific probe but not to a ß-globin intron-specific probe, I have concluded that they are probably read-through transcripts. The presence of these transcripts makes the interpretation of the nuclear run-off data more difficult. In the nuclear run-off experiments I saw a small decrease in the transcription initiation rate of the ß-39 mRNA when compared to the ß-nl mRNA. The decrease was not large enough to explain the difference in expression of the 2 mRNAs. However, if transcription initiation of the read-through transcripts is also being measured in the nuclear run-off assays, then the actual difference between transcription initiation of the ß-39 and ß-nl mRNAs could be much larger. It is therefore still a possibility that the defect in expression of the ß-39 mRNA lies in its transcription initiation. Since the presence of read-through transcripts is probably related to gene copy number, ß-39 cell lines with a low copy number could be constructed and then assayed for transcription initiation efficiency.

A further problem is that the nuclear run-off analysis presented here, although carefully standardized, represents data from 1 successful experiment. A liquid nitrogen freezer accident in September of 1985 killed all the cell lines described in this chapter. Neither the nuclear run-off assays nor the translation inhibitor experiments could be confirmed with these original cell lines. Furthermore, experiments to test the half-life of the ß-39 mRNA in the nucleus

could not be performed. This would be particularly important since there is evidence from my work and from others (Takeshita et al., 1984; Humphries et al.,1984) that the level of ß-39 mRNA which accumulates in the nucleus is reduced when compared to ß-nl.

I have hypothesized that the non-translatability of the ß-39 mRNA influences its mRNA metabolism. However, it may be that it is merely the presence of a base pair substitution, coincidentally a nonsense mutation, which influences mRNA metabolism. I do not believe that this is the case because of 2 lines of investigation. First, experimental evidence will be presented in the next chapter that missense mutations at codon 39 do not affect mRNA metabolism. Second, amber nonsense suppressor tRNA genes co-transfected with the ß-39 gene restore the level of ß-39 mRNA to that of ß-nl (Takeshita et al., 1984). I have attempted to repeat the suppression experiments by transfecting the suppressor tRNA genes into the ß-39-containing AF8 cell lines; however, I have not been able to restore the level of ß-39 mRNA to ß-nl. In a primate cell line it would be possible to infect with the SV40 virus which contains the suppressor tRNA gene (Laski et al., 1982) and this way each cell would receive the suppressor tRNA gene. In the rodent cell line that I have used the virus would not replicate. Experiments designed to optimize suppressor tRNA expression in a transient system will be discussed in Chapter 3.

In order to show that the level of ß-39 mRNA in the nucleus is reduced when compared to ß-nl , I performed Northern blots on nuclear RNA prepared from the stable cell lines. When RNA is prepared in this manner, it is difficult to show that the RNA that has been isolated is indeed nuclear. It is possible that some RNA could come from small amounts of cytoplasm which remain attached to the nuclei when the cells are fractionated. To show that I had isolated nuclear RNA, I attempted to demonstrate the existence of unspliced ß-mRNA precursors in the

nuclear fraction. I could not do this either by Northern blot or S1 nuclease analysis. It is possible that the precursors are present in such low amounts that they are not detectable by these methods. Investigators have been able to detect precursor mRNA in systems where the mRNA of interest is a predominant species in the cell (Donaldson et al., 1982; Narayan et al., 1984), unlike the ß mRNA in AF8 cells.

I was able to rule out a potential defect at a number of steps in gene expression where the defect in expression of the ß-39 mRNA could occur. The two remaining possibilities are those described above: the ß-39 mRNA is primarily defective in nuclear-cytoplasmic transport or in nuclear stability. There is no direct evidence for either of these possibilities, only that the other steps in gene expression have been excluded. Direct evidence for a defect in expression of the ß-39 mRNA could be obtained by studying transport using micronjection of *Xenopus laevis* oocytes with *in vitro* - generated mRNA. *Xenopus* oocyte germinal vesicle injection is favored over mammalian cell nuclear injection since injection into the nucleus is more easily accomplished. A cell-free approach to studying transport of this mRNA is probably not appropriate because it would be difficult to differentiate between whether the mRNA was being transported or whether it was leaking out of the isolated nuclei. Nuclear stability could be examined using the same methods that were used to examine the stability of total mRNA.

Regulation of gene expression by nuclear-cytoplasmic transport. Regulation at the level of nuclear-cytoplasmic transport or nuclear stability has been found for the expression of 2 genes. Four glucocorticoid responsive genes in rat liver (Fulton and Birnie, 1985) and the mouse DHFR gene (Leys et al., 1984) have been found to be regulated at the level of nuclear-cytoplasmic transport and nuclear stability, respectfully. The experiments for the mouse DHFR gene are more conclusive, though indirect. These investigators have shown

that the 3-fold increase in DHFR mRNA levels following growth stimulation of mouse cells results primarily from an increase in nuclear stability. By nuclear run-off analysis they showed that the transcription initiation rate of the DHFR gene was the same before and after growth stimulation. By pulse-chase experiments they showed that there was no change in the cytoplasmic stability of the DHFR mRNA. Since the gene they studied was amplified 500-fold by methotrexate selection, mRNA expression could be studied directly by *in vivo* labelling of the cells with [^{3}H]uridine followed by hybridization to DNA immobilized on nitrocellulose filters. To investigate nuclear stability, the incorporation of a pulse of [^{3}H]uridine into newly made DHFR mRNA's was compared to a pulse of [^{3}H]uridine incorporation into a reference mRNA, the 18S ribosomal RNA (rRNA) gene. They found that in resting cells the DHFR transcripts were much less stable than either the DHFR transcripts from growing cells or the 18S rRNA . They were also able to compare nuclear-cytoplasmic transport of the DHFR mRNA in resting and growing cells by [^{3}H]uridine pulses of whole cells followed by fractionation of nuclear and cytoplasmic mRNA. They found that stable DHFR mRNA is transported to the cytoplasm at the same rate in resting and growing cells. Expression of the DHFR mRNA is the first clear example of an mRNA whose expression is regulated at the level of nuclear stability. This mRNA was ideal for these studies since it is expressed at high levels. This made *in vivo* labelling of the mRNA with [^{3}H]uridine possible. Unfortunately, it would not be possible to study expression of the ß-globin gene in AF8 cells with these methods because much less ß-globin mRNA is synthesized.

Very little is known about nuclear-cytoplasmic transport of mRNA in mammalian cells. It has not been directly shown that this is a regulated step in normal RNA polymerase II gene expression. Part of the reason that so little is known about this step in gene expression is that there is no adequate assay system by which to study it. Zasloff et al.(1982) have used

microinjection of *Xenopus laevis* oocyte germinal vesicles to study transport of a variant human tRNA. They have described a tRNA mutation which interferes with nuclear-cytoplasmic transport. A more recent paper from this group (de la Pena and Zasloff, 1987) reports investigations on factors affecting nuclear-cytoplasmic transport of the Herpes simplex thymidine kinase (TK) mRNA. They studied the distribution of the TK mRNA between the nucleus and cytoplasm after microinjection of the TK gene into the nucleus of Xenopus oocytes. A surprising result was obtained when TK promoter sequences (but not coding sequences) were injected 1 hour after injection of the TK gene: there was a rapid transfer of TK mRNA from the nucleus to the cytoplasm. New RNA synthesis was not necessary for this effect. The effect seems to be specific for promoter sequences of the TK gene since linker scanning mutants in certain parts of the promoter (most notably the TATA box) abolish this effect. The data that they have presented are also consistent with a simple mass effect of nucleic acid on nuclear-cytoplasmic transport, a possibility which is not addressed. It would strengthen their specificity arguments if microinjection of RNA containing TK promoter sequences had no effect on nuclear-cytoplasmic transport or if other similar polymerase II promoters had no effect. These experiments remain to be done. Recently cell-free systems have been developed to study nuclear transport of proteins (Newmeyer et al., 1986). It was found that nuclei reconstituted from Xenopus extracts *import* nucleoplasmin in an energy-dependent fashion. Perhaps this new system with modifications will prove useful for the study of nuclear-cytoplasmic transport of mammalian mRNA's.

Nonsense mutations and nuclear mRNA metabolism. In this study I have found that a nonsense mutation, a mutation which affects the cytoplasmic event of translation, also affects intranuclear events. This implies that either there is communication between cytoplasm and nucleus in gene expresssion or that there is a mechanism which can sense nonsense

mutations in the nucleus. The communication could be mediated by an as yet unidentified small cytoplasmic ribonucleoprotein molecule (scRNP) which signals from the cytoplasm to the nucleus when a nonsense mutation is encountered. Or perhaps the communication occurs at the level of the nuclear membrane. Since the nuclear membrane is contiguous with the rough endoplasmic reticulum, it is possible that the mRNA is translated as it is transported across the nuclear membrane. If a nonsense mutation is encountered, the arrest in translation signals an as yet unidentified small nuclear ribonucleoprotein (snRNP) to stop transport of that mRNA. Or perhaps the signal is to an endogenous nuclear RNase which selectively digests the mutant ß mRNA. It is also possible that the snRNP binds to the RNA on the nuclear side of the membrane and acts as a marker for the endogenous RNase. It will be possible to study some of these events when an *in vitro* system for nuclear-cytoplasmic transport has been developed.

There is another example of an mRNA where the presence of a nonsense mutation may affect nuclear mRNA metabolism. It also supports the idea that there may be a nonsense mutation-sensing mechanism in the nucleus, and for this reason I will discuss it in detail here. Apolipoprotein-B (apo-B) is a major lipoprotein in human serum; it is present in the body in 2 forms of different molecular weight. Apo-B100 is synthesized in the liver and is used for assembly of VLDL while apo-B48 is synthesized in the intestine for chylomicron formation. They are both the product of a single gene. Recent studies (Powell et al., 1987; Chen et al., 1987) have uncovered an unprecedented tissue-specific mechanism for the synthesis of apo-B48. In the intestine, apo-B48 mRNA is synthesized by the post-transcriptional mutation of a CAA (Gln) in the coding region of apo-B100 mRNA to a stop codon, UAA. Eighty-five percent of the mRNAs with this stop codon are terminated short of the 14 kb apo-B100 mRNA, probably by using one of the cryptic polyadenylation signals. Since polyadenylation is a nuclear event (except mitochondria), a mechanism which allows for the alternative cleavage

and polyadenylation of the nascent stop codon-containing transcript may exist in the nucleus. The presence of the new stop codon may be sensed there. A different explanation is that it is the presence of the mutation-inducing enzyme on the nascent transcript which causes the alternative polyadenylation, and not the presence of the stop codon. The current data cannot be used to distinguish between this 2 possibilities.

mRNA stability studies. I have taken a novel approach to elucidating the half-life of the ß-globin mRNA's. AF8 cells, a ts RNA polymerase II BHK cell line, were selected as the parent cell line for the transfections. At the non-permissive temperature, RNA polymerase II activity in these cells declines with a half-life of 10 hours (Rossini et al., 1980). At 30 and 50 hours after shift-up to the non-permissive temperature, RNA polymerase II activity is about 12% and 3%, respectively, of baseline activity. At these time points only a small amount of transcription occurs. I compared the amount of ß-39 mRNA and ß-nl mRNA present in the cell lines at these time points to the amount present at cells before shift-up to the non-permissive temperature. Both the ß-39 and ß-nl mRNAs were stable for up to 50 hours at the non-permissive temperature. This number is in good agreement with previous estimates of the ß-globin half-life. Lowenhaupt et al. (1978) and Aviv et al. (1976) have estimated that the half-life of globin mRNA in uninduced mouse erythroleukemia (MEL) cells is greater than 50 hours. Globin mRNA half-life in DMSO-induced MEL cells is estimated to be nearly identical (Volloch and Housman,1981).

With these studies I was able to estimate mRNA half-life without the use of pharmaceutical agents. Actinomycin D is often used to poison polymerase II transcription in order to estimate the half-life of mRNA. Actinomycin D has other effects besides its well-characterized effect on polymerase II transcription, including direct effects on RNA stability. Stewart and Farber

(1968) were among the first to show this. They found that actinomycin D hastened the loss of recently labelled moieties from the nuclei of liver cells *in vivo*. It has been found that ovalbumin mRNA in chick oviduct (Palmiter and Schimke, 1973) and actin mRNA in mouse sarcoma ascites cells (Cereghini et al., 1979) are both stabilized by the addition of actinomycin D. Dreyfuss et al. (1984) have found that actinomycin D actually alters the physical state of the messenger ribonucleoproteins. They have found that the UV exposure of actinomycin D-treated HeLa cells results in crosslinking of a 38K protein to the poly A+ mRNA. The extent of crosslinking of the protein to poly A+ RNA parallels the extent of RNA transcriptional arrest. This protein is localized primarily in the cytoplasm by immunfluoresence studies with monoclonal antiboidies. It was unexpected to find this protein associated with RNA in the cytoplasm since it is widely assumed the actinomycin D has only nuclear effect. Clearly, this is not true. Since it has been sugggested that RNA instability plays an important role in the expression of another ß-globin mutant (Maquat et al., 1981), it was crucial to confirm the half-life of the ß-39 mRNA with a different method.

Determinants of mRNA stability. Determinants of mRNA stability in eukaryotic and prokaryotic cells are not well understood. Since mRNAs have different half-lives, it is likely that one of the determinants of mRNA stability is primary mRNA sequence. Recently DNA sequences in the 3' untranslated region of the gene for granulocyte-macrophage colony-stimulating factor (GM-CSF) have been identified which confer instability on mRNA (Shaw and Kamen, 1986). When these AU-rich sequences are introduced into the 3' untranslated region of the rabbit ß-globin gene, rabbit ß-globin mRNA becomes highly unstable *in vivo*. These sequences have been found to be common for a number of cytokines, lymphokines and proto-oncogenes. Complementary sequences in the mouse B2 reiterated

sequences may play a role in regulation of expression of these genes (Clemens, 1987). Sequences on the 3' end of another mRNA may play a role in determining mRNA stability. Simcox et al. (1985) have described a mutation in the major heat shock protein of *Drosphila*, hsp 70, which confers unexpected stability to the mRNA on recovery from heat shock. The hsp 40 gene is a 3' deletion mutation of the hsp 70 gene which generates both truncated mRNA and protein products. During recovery from heat shock hsp 40 mRNA is present in greater amounts than hsp 70 mRNA and the amount of hsp 40 is not affected by the addition of actinomycin D. This implies that the stability of hsp 40 mRNA is increased by the 3' deletion. Since it is known that hsp 70 mRNA is rapidly degraded during recovery from heat shock, the sequences deleted in hsp 40 mRNA may be those sequences which bind destabilizing proteins. Sequences in the 5' end may also affect mRNA stability. A sequence has been identified in the 5' untranslated region of H3 histone mRNA which confers instability on mRNA after block of DNA synthesis with hydroxyurea (Morris et al., 1986). Fusion genes between this sequence and a minigene of human ß-globin render the ß-globin gene susceptible to cell-cycle dependent degradation. These data are in disagreement with data from another laboratory (Levine et al., 1987), who find no regulatory region in the 5' end of a regulated histone gene.

Prokaryotes may have similar sequences which affect mRNA stability. Sequences which stabilize mRNA have been identified in the 3' end of gene D of bacteriophage ϕx174 (Hayashi and Hayashi, 1985). Sequences in the 5' end of the *E.coli ompA* mRNA (Belasco et al., 1986) and in the 5' end of *E.coli ermc* (Bechhofer and Dubnau, 1987) both confer increased stability on *E coli* mRNAs with shorter half lives. Recently, another type of sequence which stabilizes the upstream mRNA has been described in *E. coli.* REP sequences are inverted repeats present in many copies in the *E. coli* genome, often in the intercistronic regions of polycistronic operons. It is hypothesized that these sequences form a stem-loop structure

which acts as a barrier to the 3' to 5' exonuclease which degrades mRNA. In the *malEFG* operon, such a sequence confers differential stability on the *malE* mRNA (Newbury et al., 1987). It is more difficult to study RNA metabolism in prokaryotes than in eukaryotes since transcription, translation and RNA degradation are intimately coupled and the half-lives of mRNAs are generally extremely short. The extensive literature on prokaryotic mRNA stability will not be reviewed in this dissertation. These papers are reviewed in Higgins and Smith (1986).

Regulation of cytoplasmic mRNA stability. Cytoplasmic RNA stability has been found to be a major control point in gene expression for a number of genes. Strikingly, many of these genes are hormone-responsive genes. The RNA stability of vitellogenin,albumin, apolipoprotein VLDL II and ovalbumin is regulated by estrogen (Brock and Shapiro, 1983; Blume et al.,1987; Wolffe et al.,1985; Kazmaier et al.,1985; Wiskocil et al.,1980). Prolactin RNA stability is regulated by thyroid releasing hormone (TRH) (Lavarriere et al.,1985). Of importance to scientists using MEL cells as a model for erythrocyte maturation, DMSO induction stabilizes both actin and tubulin mRNAs (Krowczynska et al.,1985). The largest effect by an inducer on the RNA stability of an expressed gene occurs when estrogen is used to stimulate vitellogenin synthesis in Xenopus livers (Brock and Shapiro,1983; Blume et al.,1987). After estrogen stimulation, the cytoplasmic half-life of vitellogenin mRNA increases 30-fold from 16 hours to 3 weeks. The stability of the vitellogenin mRNA was measured by pulse-labelling of the RNA and hybridization to complementary DNA immobilized on nitrocellulose filters. They also showed that this effect was a reversible cytoplasmic effect of estrogen on cytoplasmic RNA stability, and have suggested that vitellogenin RNA stability is mediated directly by the action of estrogen binding protein. RNA stability is just one of the ways in which vitellogenin gene expression is regulated upon estrogen induction: estrogen also

causes a 1000-fold increase in transcription rate of the vitellogenin gene.

A distinctly unique mechanism by which the quantitative level of gene expression is regulated by cytoplasmic mRNA stability is seen for both ß-tubulin and histone mRNA. For ß-tubulin, an increase in the free subunit concentration causes a decrease in the level of ß-tubulin mRNA. This is actually a direct result of an increase in the degradation of the mRNA since enucleated cytoplasts also exhibit this phenomenon (Pittenger and Cleveland, 1985; Caron et al., 1985). The substrates for this autoregulation lie 5' to codon 18 (Gay et al., 1987) and the mRNA is required to be polyribosomal (Pachter et al, 1987). The regulation of histone gene expression has some similarities. In the normal cell cycle, histone mRNA is transcribed and is stable in S phase, and is rapidly degraded when DNA synthesis is inhibited. For experimental purposes, DNA synthesis inhibitors can be used to hasten the degradation of histone mRNAs. This specific degradation requires 2 structural components: 1) the presence of the stem-loop structure at the 3' end of the histone mRNA and 2) the presence of a fully translatable, polyribosomal histone mRNA (Graves et al., 1987; Bird et al., 1987). Since it is unclear exactly what regulates the destabilization of histone mRNA, it would be interesting to test if enucleation followed by the addition of DNA synthesis inhibitors would affect the level of histone mRNA. Other aspects of these experiments are discussed in the **Polyribosomes and mRNA stability** section of this chapter and in the **Evidence for disrupted regulation of mRNA metabolism by nonsense mutations** section in the Discussion to Chapter 3.

Mechanisms of mRNA degradation. Recent studies *in vivo* and *in vitro* have suggested that eukaryotic mRNA is degraded in a 3' to 5' fashion (Ross et al., 1986; Ross and Kobs, 1986). The stability of human H4 histone mRNA has been studied in both systems.

Following inhibition of DNA synthesis with pharmaceutical agents, the degradation products are analyzed by S1 nuclease analysis. In the *in vitro* assay, SP6 polymerase-generated histone H4 mRNAs are mixed with an *in vitro* translation mixture consisting of polysomes from K562 cells and pH5 enzymes from liver (liver ribonuclease inhibitor). Albrecht et al. (1984) were among the first to document specific nuclease activity in reticulocyte lysates. The degradation products are then analyzed at various time points. Most importantly, the differential stability of several RNAs is preserved *in vitro* (γ globin mRNA is more stable than c-myc mRNA which is more stable than histone H4 mRNA). The RNAse is cytoplasmic and is associated with polysomes. The major intermediate degradation product both *in vivo* and *in vitro* is an mRNA with a 3' terminus located at the C or U residue just 3' of the canonical stem-loop in the 3' untranslated region. A similar stem-loop structure is thought to act as a barrier to RNA degradation in the REP sequences of *E. coli* (Newbury et al., 1987), as discussed above. Prokaryotes and eukaryotes may share some similarities in their mechanisms of mRNA degradation.

Studies from our own laboratory are in agreement with these observations. Stolle et al. (1987) have examined the stability of ß-nl and ß-39 mRNAs in HeLa whole cell extracts and have determined that they have equal, long-lived stability. Furthermore, Stolle and Benz (1988) have found a factor in HeLa cells which protects ß-globin from digestion by exogenous RNases. Cell extracts contain both RNases and RNase inhibitors.

Polyribosomes and mRNA stability. It is often argued that the decrease in mRNA levels seen with mRNAs that have nonsense mutations occurs because the mRNAs are not loaded on polyribosomes and are therefore more susceptible to degradation by cytoplasmic nucleases (Maquat et al., 1981; Losson and Lacroute, 1979). These arguments are based on

observations made for prokaryotic mRNAs. The data presented in this dissertation are not consistent with this argument since they suggest that the defect is intranuclear. Data from Pachter et al. (1987) are also inconsistent with this argument. Tubulin mRNA levels are affected by the tubulin free -subunit concentration; an increase in the free-subunit concentration of tubulin decreases the level of tubulin mRNA. The polyribosome profiles presented in this paper indicate that tubulin mRNA is normally associated with polyribosomes in the cell but tubulin mRNA with a nonsense mutation is not. When the free-subunit concentration of tubulin is increased by pharmaceutical means, there is no change in the level of nonsense mutation-bearing tubulin mRNA. The mutant mRNA is actually stabilized. The mutant mRNA was not covered by polyribosomes but was actually less susceptible to degradation than the normal mRNA. "Deprotection" by dissociation from polyribosomes does not necessarily cause an increase in mRNA degradation. Other aspects of these experiments are discussed in the **Regulation of cytoplasmic mRNA stability** section in this chapter and in the **Evidence for disrupted regulation of mRNA metabolism by nonsense mutations** section in Chapter 3.

Translation and mRNA stability. I have hypothesized that translation plays an important role in the control of ß-globin mRNA metabolism. However, it is not known whether the mRNA transcribed from the transfected genes is translated. No *in vivo* studies in our laboratory have addressed this question. Data from other laboratories is contradictory. Expression of rabbit and mouse ß-globin genes cloned into the late region of SV40 has shown that substantial amounts of ß-globin protein is synthesized after infection of CV1 cells with the recombinant virus (Mulligan et al., 1979; Hamer and Leder, 1979). In both cases anti-ß-globin antibodies were used to detect the gene product. In contrast, expression of the human

α1-globin gene in an SV40-ORI vector after transfection into COS cells produced very little globin protein (Mellon et al., 1981). The vector used in the experiments described in this dissertation is different from both of these vectors. Furthermore, unlike the studies cited above, I studied expression of integrated vector DNA. It is not known if these parameters affect the efficiency of translation of the ß-globin mRNA.

Studies of globin mRNA translation in the neomycin-resistant AF8 cell lines would require monoclonal antibodies directed against purified α and ß-globin chains. These antibodies were developed by Stamatoyannopoulos et al.(1983). They have not been accessible to us for this work. Another approach to investigate whether the α and ß mRNAs are translated would be to see if they are associated with polysomes. Polysome isolation from tissue culture cells is a standard technique. Since the α and ß mRNAs are present in relatively small amounts, mRNA detection after polysome isolation may be a potential problem. A better source of polysomes would be human peripheral blood since the globin mRNAs are very abundant in red blood cells. The ß-39 gene was cloned from the genomic DNA of a patient heterozygous for Hb Lepore and ß-39. Blood could be drawn from this patient, the red blood cell polysomes isolated (Nathan et al., 1971), and then the fractions assayed for the presence of the ß-39 mRNA. Immunoprecipitation of isotopically-labelled AF8 cell protein extracts with specific anti-ß and anti-α globin antibodies may be the best way to assess whether the globin mRNAs are being translated.

If translation and mRNA decay are coupled, it is possible that mRNA levels could be reduced by the addition of translation inhibitors. The effect of translation inhibitors was tested

on one ß-nl cell line. No change in the level of steady-state mRNA was detected following the addition of either cycloheximide or NaF. However, the half-life of the ß-globin mRNA is extremely long (>50 hours), and if translation inhibitors reduce transcription of the ß-globin mRNA, no effect in the steady-state level of mRNA would be detected after only a 3 hour incubation. It would be better to assay for transcription initiation and mRNA processing after addition of the translation inhibitors. It is not clear from the experiments that I have done whether translation, translatability, or both affect accumulation of ß-globin mRNA.

There is evidence from other mammalian systems that the addition of translation inhibitors affects mRNA metabolism. The addition of emetine and cycloheximide, both peptide translocation inhibitors, stimulate the accumulation of ß-interferon mRNA by increasing the rate of transcription of the interferon gene in CHO cells (Ringold et al., 1984). Puromycin, a peptide elongation inhibitor, also has this effect. They found that both ß and γ actin transcription initiation are stimulated as well whereas α and ß tubulin are not. Another investigator (Eggerding, 1985) has found that mRNA transcribed from the early genes of adenovirus following infection of HeLa cells is stimulated to accumulate after addition of cycloheximide and anisomycin (a peptide elongation inhibitor). The accumulation is primarily due to an increased rate of transcription initiation. The steady-state level of c-myc mRNA in chicken embryo fibroblasts is also increased by the addition of cycloheximide (Linial et al., 1985). Translation inhibitors disrupt the regulated mRNA degradation of both ß-tubulin (Pachter et al., 1987) and histone mRNAs (Graves et al., 1987). It is likely that translation inhibitors have different effects on different genes.

In contrast to the data collected in mammalian systems, the data from prokaryotic systems

suggests that translation inhibitors increase degradation of newly synthesized mRNA (Varmus et al., 1971). The addition of chloramphenicol to log phase *E. coli* reduces the accumulation of *lac* mRNA. Although the half-life of *lac* mRNA is actually increased, there is also increased degradation of newly synthesized *lac* mRNA. The *suA* mutation, a polarity suppressor mutation, was found to only partially relieve the effect of chloramphenicol. Puromycin also reduces accumulation of *lac* mRNA, although in this case, the half-life of the mRNA is affected. Chloramphenicol was also found to affect the accumulation of a number of early mRNA's during infection by bacteriophage T4 (Salser et al., 1970). Schneider et al. (1978) confirmed the effect of chloramphenicol on *lac* mRNA metabolism. They then studied *lac* mRNA metabolism in the presence of kasugamycin, an inhibitor of peptide initiation. They found that the half-life of *lac* mRNA was greatly reduced in the presence of kasugamycin. From this they concluded that association with ribosomes is important for protection against mRNA degradation. This is based on the result that an inhibitor of peptide translation initiation decreases *lac* mRNA half-life. However, as noted above, some translation inhibitors which block steps in translation other than initiation also decrease *lac* mRNA half-life. Chloramphenicol, an inhibitor of peptide translocation, actually increases *lac* mRNA half-life. It is unclear whether the phenomenon studied in these experiments is related to protection of *lac* mRNA by ribosomes.

Chapter 3

The effect of nonsense and missense mutations on human ß-globin mRNA metabolism

Introduction

It is clear from the experiments presented in Chapter 2 and those of others (Takeshita et al., 1984; Humphries et al., 1984) that the ß-39 mutation is associated with a decrease in steady-state mRNA levels, probably due to a defect in intranuclear metabolism. To see if nonsense mutations at different locations and of different types produce the same phenotype, I undertook a mutational analysis of the human ß- globin gene. In making these mutations I have asked 3 questions: 1) if nonsense mutations at other positions in the gene would cause a decrease in the steady-state level of their mRNA, 2) if an ochre (UAA) mutation at ß-39 would have the same phenotype as the amber (UAG) mutation at this codon and 3) if missense mutations at ß-39 would have an effect on mRNA metabolism. In this way I could study the effect of several nonsense and missense mutations in the human ß-globin gene on globin mRNA metabolism after expression in a heterologous system.

A series of logically related mutations were constructed by site-specific oligonucleotide-directed mutagenesis (see Fig.3.1). Five nonsense mutations and 2 missense mutant human ß-globin genes were analyzed. In addition to the ß-39, I studied amber (UAG) mutations at ß-17 and ß-82, an opal (UGA) mutation at ß-37 and an ochre (UAA) mutation at ß-39. These mutations were made using the ß-nl gene as a template. I also studied two missense mutations at position 39, GAG and AAG, the codons for glutamic and lysine residues, respectively. These mutations "correct" the nonsense mutation at amino acid position 39 to translatable codons and were constructed from a ß-39 DNA template. The ß-17 and ß-37

mutations have been described in patients with ß-thalassemia and the two missense mutations have been described as normal variants. The results indicate that in the human ß globin gene the phenomenon of decreased mRNA accumulation is a general property of nonsense mutations and is independent of the type of nonsense mutation and that missense mutations have no effect on mRNA metabolism.

Materials and Methods

Cell lines. Cos-7 (Gluzman 1981) cells at low passage (<10) were obtained from the American Type Culture Collection (ATCC), Camden, NJ. Cells were maintained in DMEM supplemented with 10% fetal calf serum and 50 U/ml of penicillin, 50 µg/ml of streptomycin.

Bacterial strains. The genotype of each of the bacterial strains discussed in this chapter can be found in Table 3.1. HB101 was used for routine growth of all plasmids. RZ1032 and JM101 were used for site-specific mutagenesis.

Source of oligonucleotides. All oligonucleotides were synthesized on Applied Biosystems oligonucleotide synthesizers (Foster City, CA). Sources include: Dept. of Human Genetics, Yale University, courtesy of Dan DiMaio and Bill Summers; Integrated Genetics, Framingham, Mass., courtesy of Graham Mock; section of Immunology, Yale University, courtesy of Peter Blier and Al Bothwell.

Selection of oligonucleotides. Oligonucleotides were designed to create the following nonsense mutations: ß-17,ß-37, ß-82 and ß-39 (UAA) and these 2 missense mutations: ß-39 (GAG) and ß-39 (AAG). The exact location and type of each mutation is shown in Fig.3.1. The ß-17 and ß-82 oligonucleotides also contain a silent substitution within 6 bases of the nonsense mutations. These 2 mutations were screened for by the presence of a new restriction site.

The sequences are as follows:

ß-17

ß-nl mRNA strand sequence	5'-TGGGGCAAGGTGAACGTGG-3'
oligo 3.1	3'-ACCCCGATCCAATTGCACCG-5'

ß-82

ß-nl mRNA strand sequence	5'-CACCCTCAAGGGCACCTTTG-3'
oligo 3.2	3'-GTTGGAGATCCCATGGAAAC-5'

Amino acid substitutions at position 39

ß-nl mRNA strand sequence	5'-TACCCTTGGACCCAGAGGTTCT-3'
oligo 3.3	3'-ATGGGAACCTGGT/CTCTCCAAGAA-5'

Stop codons at position 39

ß-nl mRNA strand sequence	5'-CCTTGGACCCAGAAGTTCTT-3'
oligo 3.4	3'GGAACCTGGATTTCCAAGAA-5'

ß-37

ß-nl mRNA strand sequence	5'GTCTACCCTTGGACCCAGAGGT-3'
oligo 3.6	3'-CAGATGGGAACTTGGGTCTCCA-5'

Sequencing oligo for mutations 5' to amino acid position 51

oligo 3.5 3'-CAATACCCGTTGGGATTCCA-5'

Sequencing oligo for IVS 1

oligo 3.6 3'-CCTCTGTCTCTTCTGAGAAC-5'

Sequencing oligo for 5' untranslated region

oligo 3.7 3'-CTCGGTGTGGGATCCCAACC-5'

Purification of oligonucleotides. The oligonucleotides are supplied in a crude, partially deprotected form cleaved form support. The vial is filled with ammonium hydroxide, tightly capped, and then incubated at 55°C overnight. The vial is cooled slowly to room temperature and opened in a fume hood. The oligonucleotides are each aliquoted into 3 silanized Eppendorf tubes and dried in a Speed-Vac Concentrator. The pellets are washed twice in 0.5 ml of water and dried. The pellets are then dissolved in water. The total yield varied from 300-1200 μg/synthesis reaction.

Oligonucleotides are further purified by electrophoresis on 20% polyacrylamide Tris-borate-urea gels. The oligonucleotides are denatured and loaded in 80% formamide without tracking dye. The full-length oligonucleotide is located by UV shadowing with short wave ultraviolet light over a thinlayer chromotography plate. The gel fragment is removed with a razor blade, minced, and eluted in 0.2 M NaCl, shaking at 37°C overnight. The supernatant is precipitated with ethanol, spun, and brought up in 200 μl.

Preparation of M13 template. Either the 1.8 kb BamH1 fragment of the normal

human ß globin gene or the ß-39 gene was cloned into the BamH1 site of M13mp9. Clones that produced single-stranded (ss) DNA that was the same sequence as the globin mRNA strand were chosen for mutagenesis. Template was prepared by standard procedures (Messing, 1983).

Site-specific mutagenesis. Site-specific mutagenesis was performed according to the method of Zoller and Smith (1983) using the strains developed by Kunkel et al.(1987). Fig. 3.2 outlines the procedure.

The M13 template to be mutagenized is grown through 2 cycles of growth in the *dut ung* host, RZ1032. The bacterial overnight is diluted 1:100 in 50 ml of Luria broth supplemented with 20 µg/ml thymidine, 100 µg/ml deoxyadenosine and is grown until the cells reach a density of 4 X 10^8 cells/ml ($O.D._{600}$=0.3). The cells are spun in an RC-2B centrifuge for 10 minutes, washed in unsupplemented Luria broth, and brought up in 50 ml of pre-warmed Luria broth supplemented wiht 0.25 µg/ml uridine. The bacteria are grown for 5 minutes and then infected with 0.5 ml of M13 phage stock (approximately 10^{11}-10^{12} phage). The infected bacteria are grown overnight, and this phage stock is used to re-infect RZ1032. Template is isolated in the usual manner for M13 phages.

The oligonucleotide to be used for mutagenesis is kinased in the following manner. One µg of oligonucleotide is mixed with 2 µl with 10X kinase buffer, 2 µl of 10 mM ATP, 40 µcuries of ^{32}P-γ-ATP, q.s. to 20 µl. Add 2-4 units of T4 polynucleotide kinase. The reaction is incubated at 37°C for 60 minutes, then at 65°C for 10 minutes. The reaction is precipitated

with ethanol and brought up in 75 µl of water.

Solutions used for the mutagenesis include:

A: 0.2 M Tris-Cl pH 7.5

0.1 M $MgCl_2$

0.5 M NaCl

10 mM DTT

B: 0.2 M Tris-Cl pH 7.5

0.1 M $MgCl_2$

0.1 M DTT

C: 1 µl solution B

1µl of 10 mM dNTP solution

1 mM ATP

3 units of DNA polymerase (Klenow)

1 µl T4 DNA ligase (400 units/µl)

q.s. to 10 µl

One µg of M13 ss DNA is mixed with 8 µl of oligonucleotide and 1 µl of solution A and incubated at 55-58°C for 10 minutes. The annealed DNAs are kept at room temperature for 10 minutes. Ten µl of solution C is added to the annealed mixtures. Two µl is removed for the

gel and immediately frozen. The reaction is incubated at room temperature for 5 minutes and then placed at 15°C overnight. Two µl are removed for the gel. The samples are run on an 0.8% agarose gel with 2 µg/ml ethidium bromide. The ss form of the M13 DNA should be converted to the covalently closed ds form. Dilutions of the reaction mixtures are transformed into JM101. Plaques are picked with sterile Pasteur pipettes into 0.5 ml of Luria broth. Single-stranded or double-stranded M13 is then prepared either for DNA sequence analysis or restriction enzyme analysis, respectively.

Mutagenesis using oligo 3.3 was performed using the ß-39 gene as the template. All the other mutagenesis reactions were performed using the ß-nl gene as the template.

Analysis of mutations. The presence of a mutation in the ß-globin sequence was confirmed either by the presence of a new restriction site in miniprep M13 replicative form (RF) DNA or by direct dideoxy sequencing of the ss DNA template (Sanger et al., 1977). Minipreps of M13 RF DNA were prepared by alkaline lysis (Maniatis et al., 1982) of JM101 that had been inoculated with 1 M13 plaque. The presence of the ß-17 mutation was confirmed by the presence of a new HincII site; the ß-82 mutation was confirmed by the presence of a new Kpn 1 site. Clones which had the new restriction site were sequenced by the dideoxy method. The ß-82 mutation was sequenced using the 17-base M13 primer from BRL. The ß-17 mutation was sequenced after subcloning of a HaeIII fragment using the 17-base M13 primer form BRL. The presence of the ß-37, ß-39 nonsense mutations and the amino acid substitutions at position 39 were confirmed by direct dideoxy sequencing of the ss M13 template using oligo 3.5 as the primer.

The ß-17 mutation was sequenced in the following manner. The gel-purified 1.8 kb BamH1 fragment from ß-17 RF was digested with HaeIII. The total digest was ligated into Sma1 cut M13mp8 and transformed into JM101. Plaques were gridded. Filters were lifted (Benton and Davis,1977) and then hybridized to the kinased ß-17 oligo under stringent conditions (60°C) in the presence of tetramethylammonium chloride. Positive clones were sequenced using the commercial 17-base primer from BRL.

The ß-39 gene was sequenced from -250 through the first exon, first intron and second exon to the BamH1 site using 5 primers. The primers used were oligos 3.1,3.2,3.6,3.7 and the 17-base primer from BRL.

RF preparation. RF was prepared in the following manner. Four hundred ml of Luria broth in a 2 l flask was seeded with 4 ml of JM101 overnight. The bacteria were grown for 2 h and then inoculated with 0.2 ml of an M13 phage stock. The culture was then grown for 1-2 h. At this time chloramphenicol was added to 100 µg/ml and the culture was then grown for 3 h before harvesting. RF was harvested from the bacteria by the alkaline lysis procedure for plasmid preparation (Maniatis et al., 1982).

Subcloning. The 1.8 kb BamH1 was prepared from the RF M13 DNA by electroelution (Maniatis et al., 1982). The eluate was passed over an Elu-tip column, according to the manufacturers directions (Schleicher and Schuell, New Hampshire).

The vector used in these experiments was prepared by K. Takeshita (Takeshita el al., 1984). The 5 kb BglII fragment of the human ß-39 gene was cloned into the BamH1 site of PTL9, an SV40 vector with an SV40 origin of replication, the SV40 enhancer region and the

ampicillin resistance gene of pBR322. This version of the vector contains the pBR322 "poison sequences" of Lusky and Botchan, 1981. This vector was cut with BamH1, and the larger vector-containing fragment was gel-purified, re-ligated and transformed into HB101. In this way a PTL9 vector containing ß-globin sequences minus the 1.8 kb BamH1 fragment was prepared.

The 1.8 kb BamH1 fragments from each of the mutants was ligated to BamH1-cut vector DNA by standard procedures (Maniatis et al., 1982). Colonies were screened by colony hybridizaton (Grunstein and Hogness, 1975) with a nick-translated gel-purified 1.8 kb BamH1 fragments. Plasmid minipreps of positive colonies were digested with Nco1 to check for the correct orientation. The presence of the ß-17 and ß-82 mutations was confirmed in the plasmid by digestion with HincII and Kpn1, respectively. The presence of the ß-39 (UAA) mutation was confirmed by dideoxy ds sequencing of the plasmid using oligo 3.5.

Transfections. Plasmid DNA used in transfections was banded twice on cesium chloride. Plasmids used for transient expression studies were purified by banding in CsCl twice. Thirty µg of each of the ß-globin plasmids and 15 µg of an α–globin plasmid (pSV0α1, Mellon et al.,1981) were co-transfected into a T-150 flask of sub-confluent Cos-7 cells by the calcium phosphate method (Graham and van der Eb, 1973). After 6 h, the cells were shocked for 1 minute with 20% glycerol, washed and then fresh medium was added. An outline of this procedure can be seen in Fig.3.3.Transfection of the ß-nl and ß-39 genes was included for each experiment. When suppression of the ß-39 amber mutation was attempted, 30 µg of either pSVtT (wt) and pSVtT (Su) were co-transfected (Laski et al.,1982).

RNA isolation. Total mRNA was isolated by the method of Chirgwin et al.(1979), except that guanidine isothiocyanate was used at all steps. The RNA was stored in 70% ethanol at -20°C.

S1 nuclease analysis. S1 nuclease analysis was performed according to Meatherall et al., with these specifications. Two plasmids were used: spβA and spαA. One hundred μg of spβA and spαA were cut with BamH1 and HindIII, respectively. The total digests were phosphatased with calf intestinal phosphatase. The phosphatase was inactivated by heating to 65°C and by phenol extraction. The plasmids were precipitated with ethanol, and then stored frozen in aqueous solution. Two μg of each was kinased with T4 polynucleotide kinase and 150 μCi γ–ATP (5000 Ci/mmole, Amersham) in the presence of magnesium. To purify the kinased fragments from unincorporated γ–ATP, the DNA was ethanol precipitated, dissolved in water, and spun through a G-50 medium tuberculin syringe spun column. The yield was approximately 10^5-10^6 cpm/μl, 100 μl total volume. For each S1 reaction, $1X10^6$ cpm were used.

Hybridization of the kinased probe and RNA was performed as follows. Fifty μg of RNA was pelleted and $1X10^6$ cpm of kinased probe was added to the pellet and dried in a Speed-Vac Concentrator. Thirty μl of hybridization mix (40 mM Pipes, 1 mM EDTA, 0.4 M NaCl, 80% formamide) was added and the sample placed in a water bath at 95°C. The temperature on the water bath was then set at 55°C, and the samples were left overnight. Two hundred units of S1

nuclease were used/reaction in S1 nuclease buffer (50 mM NaOAc, pH 4.5, 0.4 M NaCl, 4.5 mM $ZnSO_4$, 20 μg/ml salmon sperm DNA). The α–globin probe samples were incubated at room temperature for 60 minutes, the ß-globin probe samples were incubated at 42° C for 30 minutes. The reactions were precipitated with ethanol and washed with 70% ethanol. The pellets were dried and dissolved in 80% formamide with xylene cyanol and bromophenol blue tracking dyes.

The samples were heated at 90°C for 2 minutes and then loaded onto a 5% polyacrylamide Tris-borate-urea gel. The bromophenol blue dye was run to the bottom. The gel was exposed overnight with a Dupont Kronex intensifying screen.

Densitometric tracing. The autoradiographs of the α– and ß-globin protected fragments were traced vertically with a Beckman DU-8 Spectophotometer.

Statistical analysis. Using the data from densitometric tracing, the amount of each of the ß-globin mRNAs was normalized to the amount of ß-nl mRNA by dividing by the amount of mutant ß mRNA. This means that the amount of ß-nl mRNA is set at 1.0. Expression of the α globin genes was treated in a similar manner. For each lane in each experiment, the ß to α ratio was calculated. The ß to α ratio for three experiments was averaged and the standard error of the mean was calculated.

Results

Purification of oligonucleotides. Fig.3.3 shows an autoradiograph of 2 ^{32}P-labelled unpurified oligonucleotides used to introduce the ß-17 (oligo 3.1) and ß-82 (oligo 3.2) mutations. The n-1 products are clearly visible. After purification, only a single band is visible.

Analysis of mutants. The mutation frequency varied from 25-85%. When mixed oligonucleotides were used, some mutations were detected at greater frequency than others. For example, with oligo 3.4, only the ß-39 (UAA) mutation was found, and not the ß-39 (UGA) mutation, although DNA from 80 plaques was sequenced.

In Fig.3.4, there is a representative agarose gel from an analysis of potential ß-17 mutations. RF was prepared and digested with HincII. A mutation is indicated by the presence of a new HincII restriction site. The mutation frequency here was 25%. Fig.3.5 shows a similar analysis of potential ß-82 mutations.

Figs.3.6-3.8 depict the dideoxy sequence analysis used to screen for the presence of the mutations. Fig. 3.6 shows the results of screening for missense mutations at ß-39. In this case, the mutation occurs where there is the absence of an A. The frequency of mutation is about 60%. Fig.3.7 shows the results of screening for the UAA and UGA termination codons at ß-39. The mutation frequency is about 30%. Fig.3.8 shows the results of screening for the ß-37 mutation. The mutation frequency is about 85%.

DNA sequence analysis. In Figs.3.9-3.11 are shown the DNA sequences of the

regions in the human ß globin gene that were mutated. In Fig.3.9 is shown the sequence of the ß-82 and ß-17 mutations. In Fig.3.10 is shown the sequence of the 2 missense mutations. In Fig.3.11 is shown the sequence of the ß-39 (UAA) mutation and in Fig.3.12 the sequence of the ß-37 mutation.

DNA sequencing of the intact ß-39 gene confirmed previous results that the only mutation in that gene is the ß-39 mutation (Takeshita et al., 1984).

Transfections. Fig.3.1 shows the 7 mutations that were tested in the COS transient expression system. As indicated, all mutations were derived from the ß-nl template except for the 2 missense mutations at codon 39, which were derived from the ß-39 (UAG) template. The mutations are located throughout the first two exons of the human ß globin gene. The type of mutation that occurs at each location is also indicated.

Fig.3.13 is an example of the results of an analysis of the expression of each of the ß-globin genes. The relative expression of the ß-mRNA's was determined by S1 nuclease analysis of total RNA from transfected COS cells. This figure indicates that the ß-nl, ß-39 (GAG) and ß-39 (AAG) genes all express approximately the same level of mRNA. In contrast, each gene which bears a nonsense mutation expresses the ß mRNA in reduced amounts. The α globin gene was co-transfected to monitor plate-to-plate changes in transfection efficiency. The α globin mRNA levels are relatively constant in this experiment. The double band seen in the α globin S1 nuclease digestion is an S1 nuclease artifact, probably due to breathing at the A-T rich end of the DNA-DNA hybrid. To quantify these results, the autoradiographs from 3 separate experiments were traced on a Beckman spectophotometer.

The results are shown in Fig.3.14. The average ß to α ratio plus-or-minus the standard error of the mean is indicated. In each case where there is a nonsense mutation, the level of ß mRNA is reduced when compared to the level of ß-nl mRNA. Missense mutations have no effect on mRNA levels. Correcting the amber nonsense mutation at codon 39 to missense mutations brings the level of mRNA that accumulates back to normal. There is some variability in the levels of expression between mRNA's within each group. For example, the average amount of ß-39 (UAA) mRNA that is produced is greater than the average amount of ß-39 (UAG) mRNA that is produced. However, since there is much overlap in the error bars between these two it is difficult to assess whether they are indeed different. For the nonsense mutations the variability is probably not related to the type of codon or to its relative position in the transcript.

Table 3.1

E. coli strains

BW313	HfrKl16 PO/45 [*lysA* (61-61)], *dut1*, *ung1*, *thi1*, *relA1*
CJ236	*dut1*, *ung1*, *thi1*, *relA1*/pCJ105 (Cm^r)
HB101	*F-*, *hsdS 20* (r^-_B, m^-_B), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20 (Smr)*, *xyl-5*, *mtl-1*, *supE44*, *λ–*.
JM101	*Δlacpro*, *supE*, *thi*, *F'traD36*, *proAB*, *lacIqZΔM15*
RZ1032	as BW313, but Zbd-279::*Tn10*, *supE44*

Fig.3.1. Origin, location and type of mutations in the human ß globin gene. A map of the mutations analyzed is shown. Nonsense mutations at ß-17, ß-37 and ß-39 (UAA) and ß-82 were constructed from the ß-nl template. The ß-nl codon at each position is shown and the nonsense mutations are enclosed in boxes. The missense mutations, which are circled, were constructed from the ß-39 amber (UAG) mutant be site-directed mutagenesis. IVS, intervening sequence. The hatched bars indicate the 5' and 3' untranslated sequences and the closed bars indicate the exons.

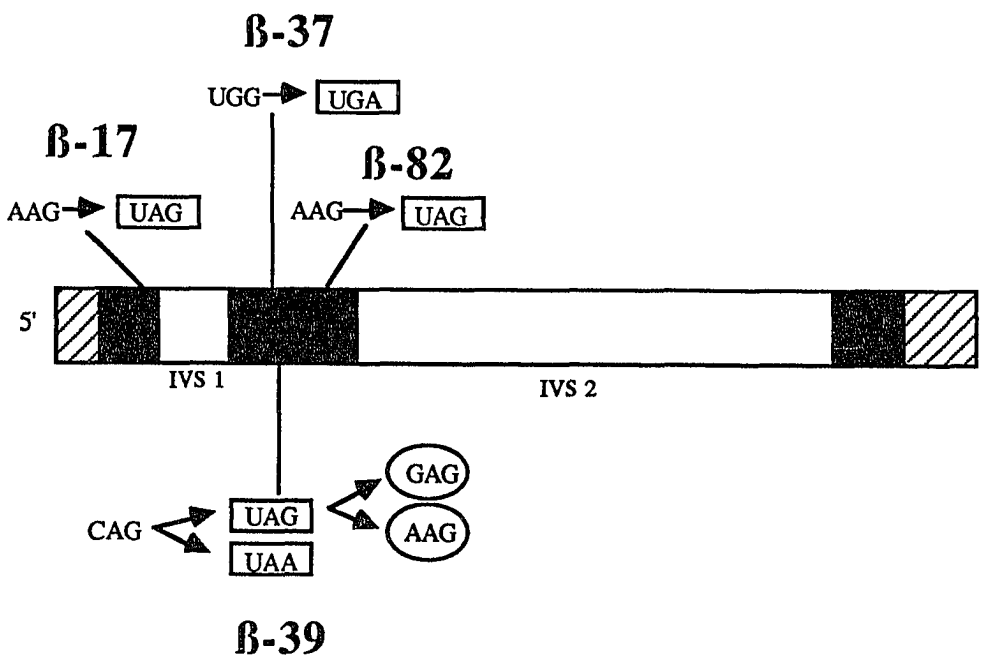


Fig.3.2 Outline of the procedure for site-specific mutagenesis. M13 phage was grown in a repair-deficient *E.coli* strain which allows uracil to be incorporated into the template DNA. The synthetic oligonucleotide is annealed to the template DNA and then extended in the presence of deoxynucleotides, T4 DNA ligase and DNA polymerase (Klenow). The reaction mixture is then transformed into JM101, a non-repair deficient host, and the mutants identified.

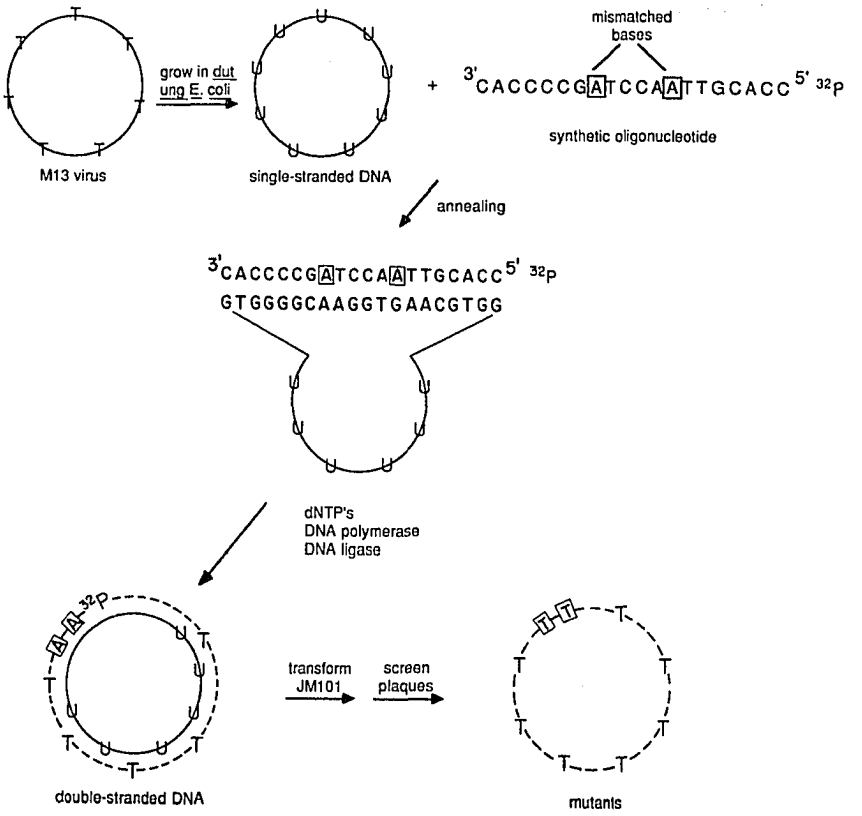


Fig.3.3 Purification of oligonucleotides. Shown are 2 unpurified ^{32}P-labelled oligonucleotides, the ß-17 (oligo 3.1) and the ß-82 (oligo 3.2). The top band is the full-length oligonucleotide; the n-1 products are clearly visible. After purification, only 6a single band is visible.

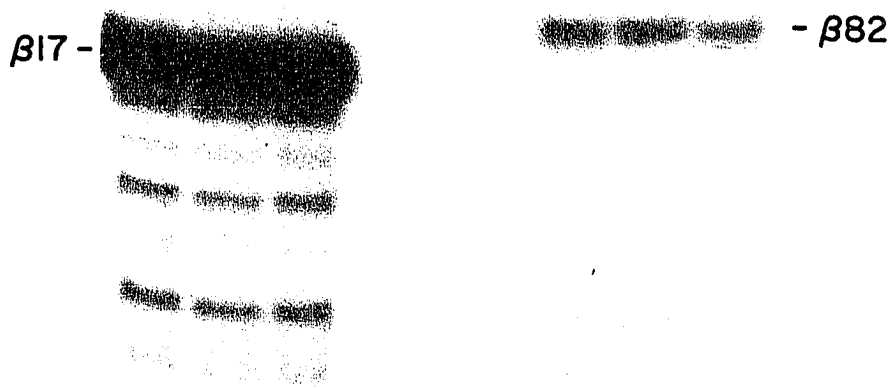


Fig.3.4 Screen for ß-17 mutations by restriction endonuclease analysis.

Six minipreps of M13 RF were analyzed for the prsence of a new Hinc II site. The new band, indicated by the arrow, is present in minipreps 1,3 and 4.

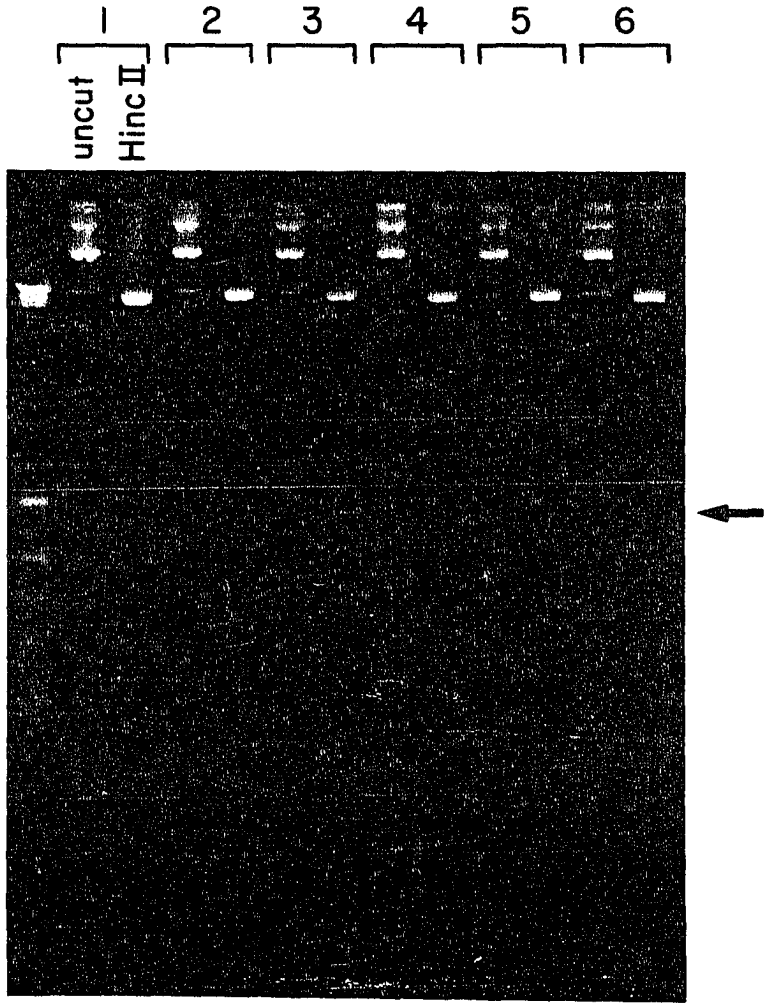


Fig.3.5 Screen for ß-82 mutations by restriction endonuclease analysis.

Four minipreps of M13 RF were analyzed for the presence of a new Kpn site by Cla-Kpn double digests. The new band, indicated by the arrow, is present in lanes 1,2 and3.

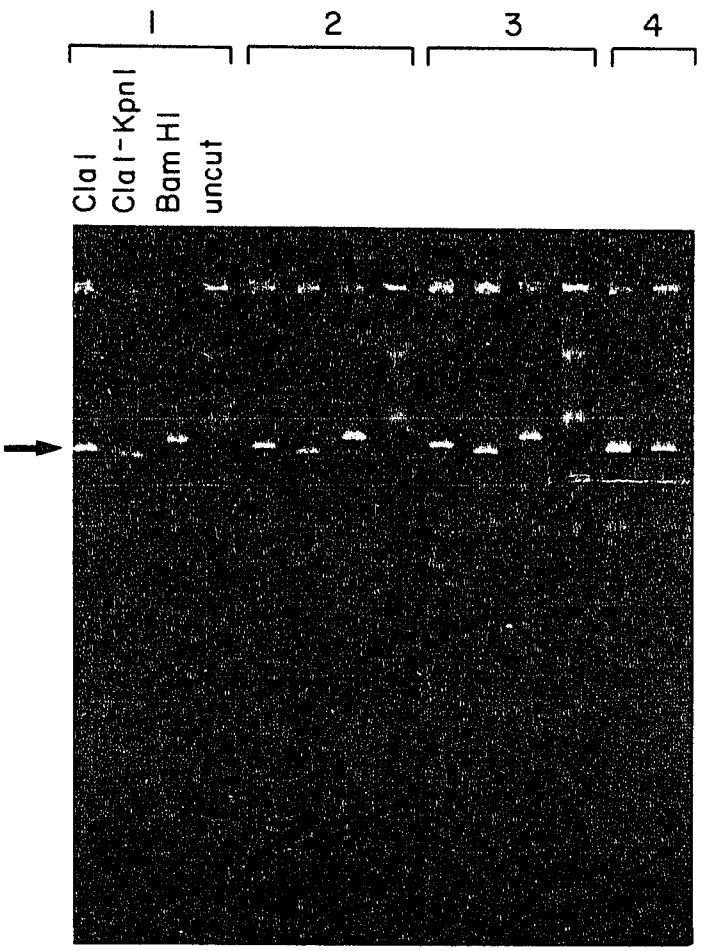


Fig.3.6 Screen for ß-39 missense mutations by dideoxy DNA sequencing.

Shown are A tracks; the mutation occurs where there is the absence of an A. Mutations occur in lanes 1,5,6,7,8,9,10 and12.

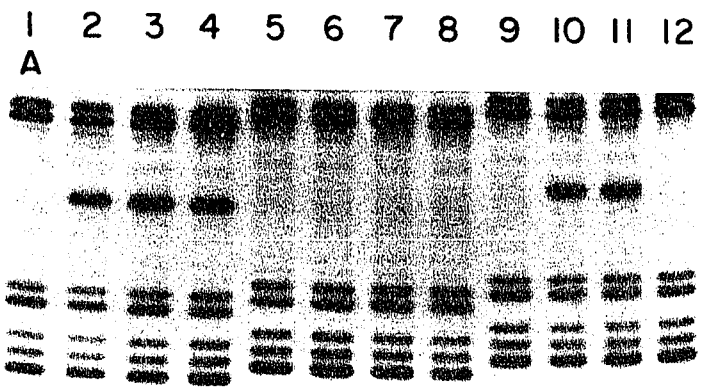


Fig.3.7 Screen for ß-39 nonsense mutations by dideoxy DNA sequencing.

Shown are C and T tracks; the mutation occurs where a T is substituted for a C. Mutations occur in lanes 3 and 6.

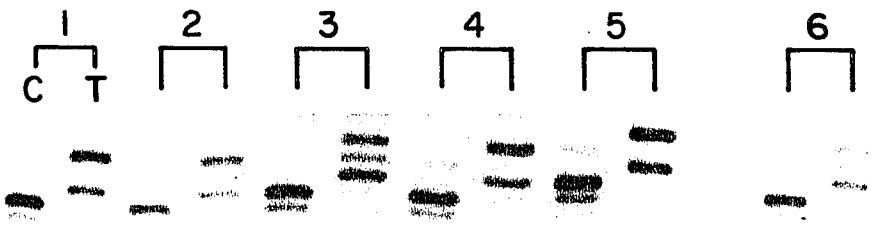


Fig. 3.8 Screen for ß-37 mutations by dideoxy DNA sequencing. Shown are T tracks; the mutation occurs where there is a doublet of T's. Mutations occur in lanes 1,2,3,5,6,7,8,9,11 and 12.

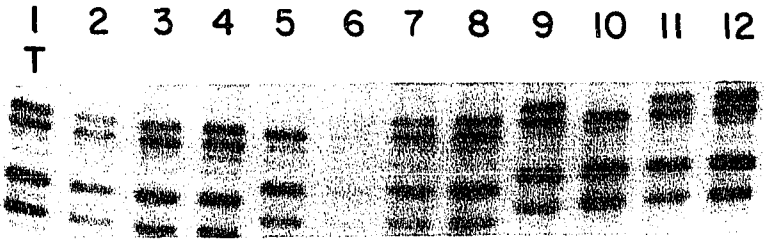


Fig.3.9 DNA sequence of the ß-82 and ß-17 mutations. Shown is the DNA sequence of the mutated region for each of these mutations. The mutated bases are indicated.

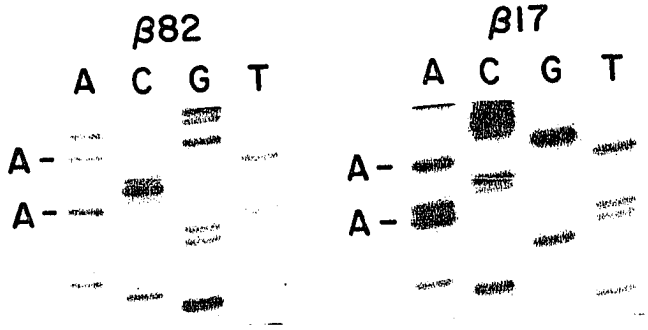


Fig.3.10 DNA sequence of the ß-39 missense mutations (AAG and GAG).

Shown is the DNA sequence of the mutated region for each of these mutations. The mutated bases are indicated.

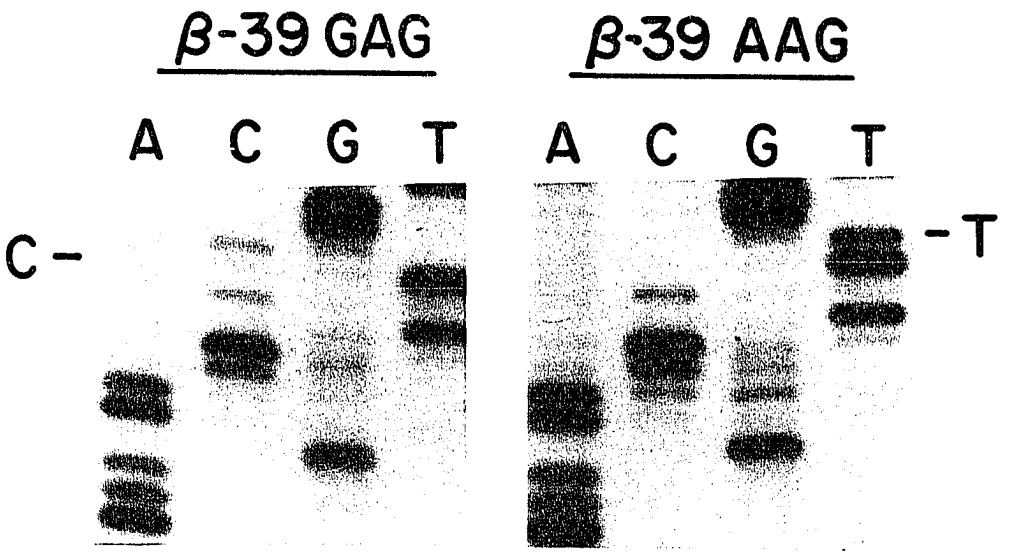


Fig.3.11 DNA sequence of the ß-39 (UAA) mutation. Shown is the DNA sequence of the mutated region; the mutated bases are indicated.

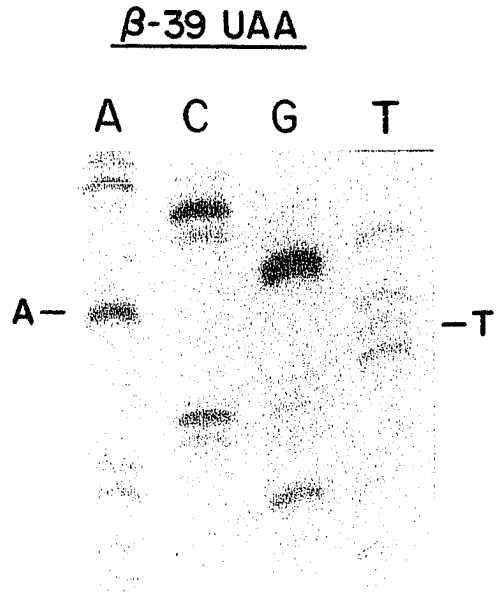


Fig.3.12 DNA sequence of the ß-37 (UGA) mutation. Shown is the DNA sequence of the mutated region; the mutated base is indicated.

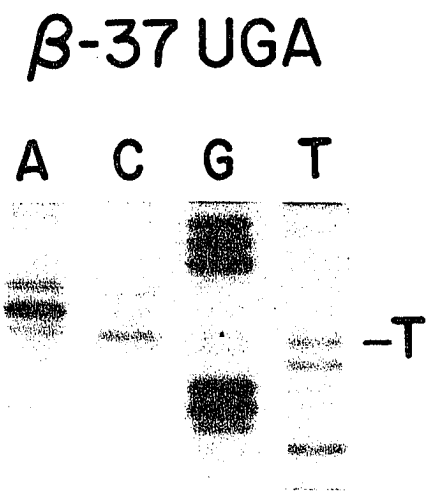


Fig.3.13 Relative expression of nonsense and missense mutant ß-globin mRNA. The upper gel depicts an autoradiograph of the S1 nuclease analysis of the ß-nl, nonsense and missense mutants, as indicated. In all cases the expected protected ß-globin fragment of 209 base pairs was generated. The lower gel depicts an autoradiograph of the S1 nuclease analysis of the α-globin mRNA. The α-globin gene was co-transfected with each of the ß-globin constructs to control for transfection efficiency.

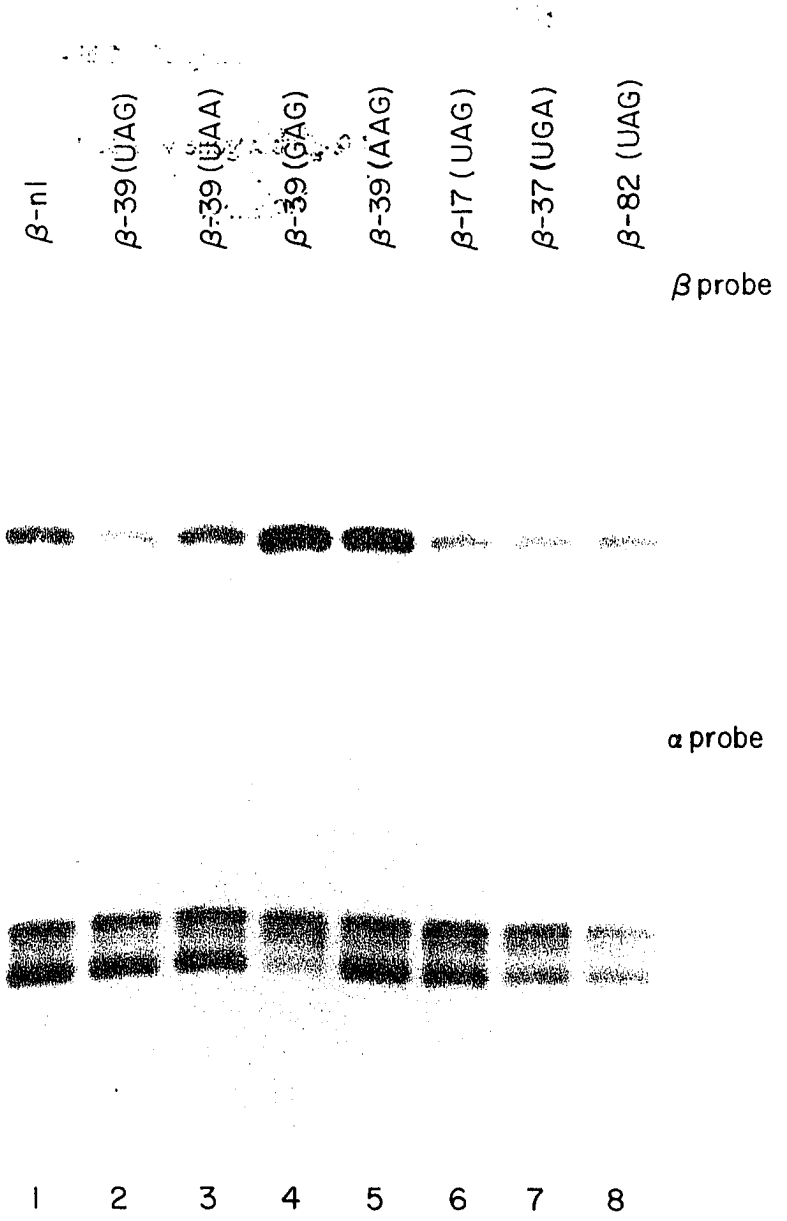
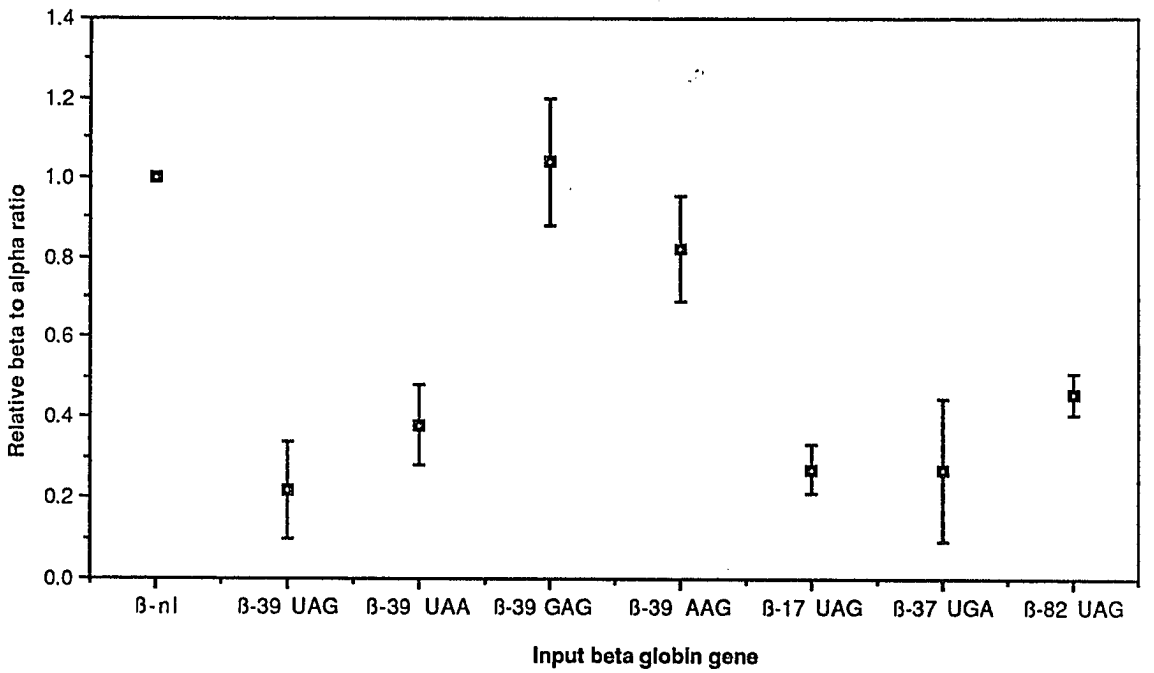


Fig.3.14 Quantitation of the relative expression of nonsense and missense mutant ß-globin mRNA. A graph of the relative expression of the nonsense and missense mutant ß-globin mRNAs is shown. Autoradiographs of the S1 nuclease analysis of the ß-globin and α-globin mRNAs were traced by densitometry. The ß-to-α ratio (ß/α) was calculated for each construct and then, within each experiment, normalized to the amount of normal ß-globin mRNA. The amount of wild-type ß-globin mRNA is designated as 1.0. The graph indicated the mean plus-or-minus the standard error of the mean for three experiments.



Discussion

Summary. I have studied the effect of nonsense mutations in the human ß globin gene on mRNA metabolism. I constructed amber (UAG) mutations at codons 17 and 82, an opal (UGA) mutation at codon 37 and an ochre (UAA) mutation at codon 39. In addition I have corrected the UAG at the ß-39 gene to two missense mutations AAG and GAG. The mutations at ß-17, ß-37, and ß-39(UAG) have been described in patients with ß° thalassemia. This series of mutants allowed me to ask 1) if nonsense mutations at other positions in the gene would express mRNA in reduced amounts, 2) if the ochre (UAA) mutation at ß-39 would have the same phenotype as the amber (UAG) mutation at this codon, and 3) if the missense mutations at ß-39 would have an effect. Assays of steady-state mRNA levels were performed by S1 nuclease analysis of mRNA from Cos-7 cells transfected with the various mutants. I have found that the ß globin mRNA's transcribed from genes with nonsense mutations accumulate to reduced levels while the genes "corrected" with missense mutations at codon 39 make normal levels of ß globin mRNA. This suggests that in the region of the gene we have studied the phenomenon of reduced mRNA accumulation is a general property of nonsense mutations.

Our data suggest strongly that nonsense codon mutations in the human ß globin mRNA give rise to a defect in ß globin mRNA accumulation. The defect seems to be independent of type (amber vs. ochre vs. opal) and the location of the nonsense mutation. The absolute level does not correlate with the position of the nonsense mutation in the gene. It is interesting that 2 different nonsense mutations, amber (UAG) and ochre (UAA) at amino acid postion 39, give rise to similarly reduced levels of ß globin mRNA.

Nonsense mutations and globin mRNA metabolism. Several lines of evidence support the notion that reduced ß globin mRNA accumulation is a result of the presence of the nonsense mutation in the ß globin mRNA. The reduction in steady-state levels of mRNA is seen with all 5 of the nonsense mutations but not with either of the missense mutations, even when the missense mutation is inserted at the same nucleotide. This implies that mRNA accumulation is not affected merely by the occurence of a mutation at a particular location; rather the occurence of a translation termination mutation is crucial to the reduced mRNA levels. Since the missense mutant genes were constructed using the ß-39 (UAG) as a template, this experiment confirms that decreased mRNA accumulation is due to the presence of the nonsense mutation and not to any other mutation occurring in that gene, or any aberrant features of that construct. Direct DNA sequence analysis has demonstrated that the amber (UAG) mutation is the only mutation occurring in the ß-39 gene.

Previous studies have shown that the non-translatable ß globin mRNA transcribed from this vector initiates, splices and terminates correctly both *in vivo* and *in vitro* (Humphries et al., 1984 or see Chapter 2) These experiments were performed by S1 nuclease analysis either of mRNA isolated from the peripheral blood of patients with ß thalassemia or of mRNA isolated from stable cell lines expressing the ß-39 mRNA.

There is a paucity of studies of nonsense mutations in mammalian genes. For ß globin, only a handful of the nonsense mutations have been described. Low levels of accumulation of nonsense mutant ß globin mRNA has been reported for both the ß-17 (Chang and Kan, 1979), ß-39 (UAG) mutants (Moschonas et al., 1981; Orkin and Goff, 1981; Trecartin et al., 1981; Gorski et al., 1982; Benz et al., 1978) and the ß-43 (Atweh et al., 1988) mutants in mRNA isolated from the peripheral blood of patients with ß° thalassemia. mRNA accumulation *in*

vivo for the ß-37 mutant has not been investigated (Kazazian et al., 1984). *In vivo* the level of ß-17 and ß-39 mRNAs that accumulates is reported to be 15% and 5% of normal, respectively. The amount of ß-43 in patients peripheral blood is less then 3% of normal. The *in vitro* data mimic the *in vivo* patient data to the extent that, for all mutants tested, reduced levels of nonsense mutant mRNA are observed. However, the exact percentage of ß-nl mRNA that accumulates is not identical *in vitro*. The experiments presented here indicate that the ß-17 mRNA accumulates to about 30% of normal (15% *in vivo*) and the ß-39 (UAG) to about 20% of normal (5% *in vivo*). Atweh et al. (1988) have studied that expression of the ß-43 *in vitro* and have found that the mRNA is present in reduced amounts, about 35% of normal. The ß-82 mutant accumulates to about 50% of normal *in vitro*.

There are no *in vivo* data on mRNA levels for the missense mutations, although both mutations have been described *in vivo* (Kendall et al., 1977; Brimhall et al., 1975). Neither mutation is associated with thalassemia. The ß-39 (AAG) mutation has been identified in a voluntary screening program as hemoglobin Alabama and is not associated with any hematologic abnormality. The ß-39(GAG) mutation has been identified as hemoglobin Vaasa in a Finnish family. This ß chain is mildly unstable and is associated with a very mild hemolytic anemia in the heterozygote.

A nonsense mutation has also been described in the third exon of the α-globin gene, α–116, UAG (Liebhaber et al., 1987). The total number of amino acids in α globin of normal length is 141 amino acid residue. The α-116 mRNA accumulates in normal amounts. This is not unexpected since a ß globin mRNA with a presumed nonsense mutation in the third exon (Hb McKees Rocks; Winslow et al., 1976) also accumulates to normal levels.

Moschonas et al.(1981) were among the first to test the expression of the ß-39 gene in a transient expression system. They compared the expression in HeLa cells of the BglII fragments from the human ß globin gene and from the ß-39 gene cloned into a pBR328-SV40 vector. They saw no difference in expression of the two genes, in contrast to my results and to those of Takeshita et al.(1984) and Humphries et al.(1984). The reason for this is not known. It is not likely that this is simply the result of the use of different expression vectors since Atweh et al. (1988) have successfully used yet a different vector (πSVplac) to compare ß-43 mRNA levels to ß-nl in a HeLa transfection system.

It has been hypothesized that the primary defect in expression of another ß°-thalassemic mRNA is its instability (Maquat, L.E. et al., 1981). A single base pair deletion has been described at codon 44 which produces an in-phase opal (UGA) translation termination codon at amino acid position 60 (Kinniburgh, A.J. et al., 1982). mRNA metabolism studies were performed on nucleated bone marrow cells from a patient with homozygous ß°-thalassemia. It was determined that in a 30 minute incubation with actinomycin D, at least 50% of the normal-sized mutant ß mRNA was degraded. Less than 10% of the ß-nl mRNA was degraded in this time period. From this they concluded that the mutant ß mRNA was unstable. Since nuclear and cytoplasmic mRNA fractionation studies were not done, it is possible that they were observing mRNA instability in the nucleus. The data from our laboratory and theirs may be in agreement.

Polarity. Study of the effect of nonsense mutations on mRNA metabolism in prokaryotes has led to the description of a phenomenon known as polarity (Morse et al., 1969; Adhya and Gottesman, 1978). Polarity refers to the effect that a nonsense mutation has on

mRNA stability. By pulse-chase experiments it has been shown somewhat indirectly that the more 5' the nonsense mutation, the more unstable is the mRNA. Although the mechanism of polarity is not completely understood, the simultaneous occurence of both transcription and translation in the prokaryotic cell is thought to be a necessary requirement.

A polar effect of nonsense mutations on mRNA metabolism has been described for 3 mRNAs in yeast. *URA1*, *URA3*, and *CYC1* mRNAs with nonsense mutations acumulate in reduced amounts. Amber mutations in the 5' end of the yeast *URA3* gene have a greater effect on mRNA stability than mutations at the 3' end (Losson and Lacroute, 1979). This is also true for ochre mutations in the yeast *URA1* gene (Pelsy and Lacroute, 1984). Nonsense mutations in the CYC1 mRNA have a polar effect on mRNA metabolism, even when one of the mutations is an ochre and one is an amber (Zitomer et al. 1979). In each of these cases they related their observations to mRNA stability but did their experiments in such a way that they would not detect a defect at the level of intranuclear metabolism.

A series of nonsense mutations in mouse μ-mRNAs has been studied by Baumann et al. (1985). They found reduced mRNA levels for 17 μ-mRNAs with either point or frameshift mutations. In general, nonsense mutations near the 5' end of the mRNA have a greater effect on the level of mRNA than mutations more 3. There was no direct linear correlation between position in the gene and mRNA level, however. In addition, there were apparent exceptions. DNA from only 5 of the mutant genes has been sequenced and their nonsense mutations confirmed. There is no DNA sequence analysis for the remaining 12 mutant genes; the presence of nonsense mutations was inferred because truncated peptides are produced. Final interpretation of these data requires DNA sequence confirmation for all 17 mutations.

For the ß globin mRNAs it has been difficult to asses whether nonsense mutations have a polar effect on mRNA metabolism. Indeed from the *in vivo* data, it has been hypothesized that there is a reverse polarity, since the amount of ß-17 mRNA is greater than the amount of ß-39 mRNA (see **Nonsense mutations and globin mRNA metabolism** in this chapter.) From our *in vitro* data, it cannot be said whether there is a polar effect of nonsense mutations on human ß globin mRNA metabolism in transfected tissue culture cells. The number of samples are too few to include or exclude this hypothesis.

In order to evaluate whether or not there is polarity of nonsense mutations in human ß globin mRNA metabolism, it may be crucial to study expression of a nonsense mtuation in the third exon. All of the mutations studied thusfar have been in the first 2 exons. These mutations may not be distributed far enough apart in the mRNA to see polarity if it exists. A ß globin gene with an ochre (UAA) mutations at amino acid position 121 has been cloned from a patient with ß-thalassemia (Kazazian et al., 1986). The presence of this mutations in the DNA also disrupts the EcoR1 site. There are no *in vivo* studies on the expression of the ß-121 mRNA. Since the mutation is in the third exon, subcloning the relevant portion of the gene into the expression vector is difficult. A good subcloning strategy is as follows. The 2.5 kb SnaB1-Xba piece of the ß-121 is ligated in a 3-way ligation to the appropriate vector sequences. In this way a ß globin gene identical to the other genes except for the ß-121 mutation can be studied in a heterologous transfection system.

Mechanism. We have found, then, that mutations in the human ß globin gene which prematurely terminate translation also affect mRNA metabolism. One possible explanation is that since the mRNA is not completely translated, it is not protected by ribosomes and is

therefore susceptible to endogenous RNase digestion. Previously published reports from our lab (Takeshita et al., 1984) and from others (Humphries et al., 1984) have demonstrated that, at least for the ß-39 (UAG) mutant, the decrease in mRNA accumulation is not a result of cytoplasmic instability. These results have been confirmed in stable cell lines which contain either the ß-39 (UAG) or normal ß globin genes (see Chapter 2). The defect in metabolism of nonsense mutant mRNAs therefore probably resides in the nucleus. One potential mechanism is that there is feedback from translation on intranuclear events and that the presence of a premature termination codon during translation in the cytoplasm serves as a signal to inhibit transport of the specific mRNA out of the nucleus. It is also possible that the presence of the premature termination codon can be sensed within the nucleus itself. These possibilities are discussed more extensively in Chapter 2.

Nonsense mutations in other eukaryotic mRNAs. One of the first nonsense mutations to be described in a non-fungus eukaryote was in the heavy chain of myosin of the nematode, *Caenorhabditis elegans* (Epstein et al., 1974). The mutation was induced by ethyl methanesulphonate and creates semi-paralyzed nematodes. The presence of the mutation was inferred by the appearance of a shortened myosin heavy chain in Laemmli gels of protein extracts, about 3% shorter than the total length of the normal protein. Concurrently, Cowan et al. (1974) described a non-secreting mouse myeloma cell line which synthesized a heavy chain shorter than the normal heavy chain by 700 daltons. Techniques such as DNA sequencing or *in vitro* suppression were not available at that time to confirm the presence of the mutations. mRNA studies have not been done.

Summers et al. (1975) described several nonsense mutations in the thymidine kinase (TK) gene of the Herpes simplex virus. This protein is ideal for such studies since TK activity can

be selected for and selected against by pharmacological manipulations. A rapid assay is also available for TK activity (Cremer et al.,1978). They identified amber (UAG) and opal (UGA) mutations which gave rise to TK proteins of lower molecular weight (Cremer et al.,1979). These mutations were suppressible by the appropriate yeast suppressor tRNA's in *in vitro* translation reactions. *In vivo* suppression of the amber (UAG) mutation was achieved by the co-infection of an SV40 viral vector bearing an amber suppressor tRNA (Summers et al.,1983).

Several nonsense mutations have been described in other viral genes. Gesteland et al. (1977) described amber and ochre mutants of the ND1 protein of an adeno-SV40 virus hybrid. An amber mutation was created near the 3' end of the T-antigen gene of SV40 (Rawlins and Muzyczka,1980; Rawlins et al.,1983). T-antigen bearing this mutation loses its ability to autoregulate its synthesis. Six nonsense mutations have been described in the NS1 gene of influenza A virus (Parvin et al.,1983). Truncated peptides were detectable following expression of these mutant genes. A nonsense mutation has been created in the open reading frame E2 of bovine papillomavirus which reduces the transformation efficiency of viral DNA in mouse C127 cells (DiMaio,1986). It is not known whether the truncated peptide is synthesized (DiMaio, personal communication).

Two naturally occurring nonsense mutations have recently been described in *Drosophila melanogaster* (Karlik et al.,1984; Martin et al.1985). Opal (UGA) mutations have been described in the alcohol dehydrogenase gene and the actin genes. Truncated peptides were detectable in both cases.

A candidate truncated peptide has been found in reticulocytes from a patient with the ß-121

mutation; it makes up between 0.05% and 0.1% of the total globin protein (Adams et al., 1986). Other than the ß-121, no truncated peptides have been found in reticulocytes from patients with nonsense mutations in their ß globin genes (Bunn and Forget, 1986). Whether peptides are synthesized and degraded rapidly is not known.

In most of the investigations involving the expression of genes with nonsense mutations that I have described, the truncated peptide was detectable. The truncated peptide in these cases appears to be made in amounts equivalent to the full-length protein. Although mRNA studies were not performed, it is possible that in these cases the amount of mutant mRNA which accumulates is similar to the amount of normal mRNA which accumulates. Another possibility is that less mutant mRNA accumulates but it is translated more efficiently.

Nonsense mutations in other human mRNAs. Naturally occurring nonsense mutations have been found in several other human genes. Nonsense mutations have been described for LDL receptor genes in patients with hyperlipidemia (Lehrman et al., 1985; Lehrman et al., 1987). *In vivo* mRNA studies on one of these genes has not demonstrated reduced levels of LDL receptor mRNA (Lehrman et al., 1987). A nonsense mutation has also been described in the human protein C gene (Romeo et al.,1987). The presence of this mutation causes hereditary thrombophilia. α_1-antitrypsin deficency can be caused by a frameshift mutation that creates an in frame stop codon (Nukiwa et al. 1987). mRNA and detailed protein studies have not been done. It is possible that the effect that we have observed occurs only with mRNAs that accumulate as a major species in the cell. Further studies remain to determine if our observations for nonsense mutations in the human ß globin mRNA are a general property of nonsense mutations in eukaryotic genes.

Daar and Maquat (1988) have described nonsense mutations in the human gene for triosephosphate isomerase which decrease the level of steady-state mRNA. The actual mechanism for this remains to be elucidated.

Evidence for disrupted regulation of mRNA metabolism by nonsense mutations. It is clear that the introduction of a nonsense mutation into a ß globin mRNA decreases the steady-state level of the mRNA. As discussed in Chapter 2, this is probably due to either a primary defect in nuclear-cytoplasmic transport or a primary defect in intranuclear stability. In either case, the fate of the mRNA is to be degraded. There are two examples, however, where normal mRNA metabolism is disrupted by the presence of a nonsense mutation and the end result is not degradation of the mRNA. In both cases the mRNA is actually stabilized by the presence of a nonsense mutation. Tubulin mRNA is degraded in the cytoplasm in response to an increase in the free-subunit concentration of the tubulin protein (Pittenger and Cleveland, 1985; Caron et al., 1985). The introduction of a nonsense mutation at amino acid 26 (tubulin is 444 amino acids long) blocks this autoregulation (Pachter et al., 1987); the mRNA is not degraded in response to a rise in the free -subunit concentration of tubulin. This nonsense mutation-containing mRNA is not found on polyribosomes and the authors use this to argue that a polyribosomal location of the mRNA is necessary for tubulin autoregulation. Nonsense mutations have a similar effect on the metabolism of histone mRNA. Histone mRNAs with nonsense mutations are not degraded in a cell-cycle specific manner (Graves et al., 1987). These studies suggest that nonsense mutations in eukaryotic mRNAs have no single effect on mRNA metabolism. In some cases, disruption of regulation of normal mRNA metabolism occurs. Metabolism of these 2 mRNAs is also discussed in the section on **Polyribosomes and mRNA stability** and the section on **Regulation of cytoplasmic**

mRNA stability in Chapter 2.

mRNA secondary structure. It is clear from my work that I am not studying an effect of mRNA secondary structure since nonsense mutations but not missense mutations at the same position have an effect on mRNA metabolism. Yet it remains an interesting possibility that secondary structure of eukaryotic mRNAs influences mRNA metabolism. Very little is known about mRNA secondary structure and regulation of mRNA metabolism. Perhaps the most convincing example is the requirement for the stem-loop structure at the 3' end of histone mRNA for correct 3' end processing (Birchmeier et al., 1983) and cell cycle regulated synthesis (Graves et al., 1987). In fact, this is one of the only portions of an mRNA molecule for which there is a definite secondary structure. Although two groups have have elucidated RNase S1 and T1 sensitive regions of rabbit and mouse ß globin mRNA it is not known (and seems unlikely) that there is a functional correlation for these regions (Pavlakis et al., 1980; Albrecht et al.,1984). Furthermore, no mutant mRNAs were analyzed in this system.

More information could be gleaned from a thorough study of missense mutations in the human ß globin gene. Fortunately, nature has done this for us. There are over 200 known naturally occurring amino acid substitutions in the human ß globin gene (Bunn and Forget, 1986). These are known as hemoglobin variants since only 50% of the amino acid substitutions are associated with a clinical abnormality. Amino acid substitutions have been detected at 126 out of the 146 residues. Among these variants, only a handful are associated with ß-thalassemia and most of these produce unstable ß chains. There are only 2 variants from which we may learn about the effect of mRNA secondary structure on mRNA metabolism. These are Hb Vicksburg (ß75→0) and Hb North Shore (ß134 Val→Glu). The former is known to be associated with reduced mRNA levels (Adams et al., 1981). However,

the gene has not been cloned and it remains to be determined whether there is another mutation in *cis* to this mutation that causes the phenotype. Hb North Shore mRNA levels have not been studied but this mutation is associated with a decrease in ß chain production (Smith et al., 1983). This gene remains to be cloned and its expression thoroughly studied. Hb E (ß26 Glu → Lys) was also once thought to be similar to Hb Vicksburg and Hb North Shore in this respect, but it has since been shown that this mutation causes a defect in mRNA splicing (Orkin et al., 1982) . There may not be much of an effect of mRNA secondary structure on mRNA metabolism for ß globin since only 2 out of 232 known ß chain variants are candidates for this phenomenon. However, since 65% of the possible variants remain to be described it may be that one of them causes a change in mRNA metabolism. It is likely that a significant mutation would be detected since it would be associated with hemtologic abnormalities.

Nonsense suppression. Takeshita et al. (1984) performed a crucial experiment in order to test the hypothesis that translatability influences mRNA levels. They co-transfected normal and suppressor tyrosyl tRNA genes from Xenopus with the ß-nl and ß-39 genes. The tRNA genes were cloned into the late region of an SV40 virus/pBR322 construct (Laski et al., 1982) and therefore could replicate in COS cells. In the presence of the suppressor gene but not the normal tRNA gene, the ß-39 mRNA levels were restored to normal. The suppressor gene had no effect on the level of ß-nl mRNA produced. When the ß-39 mRNA was able to be translated to its full-length by the addition of a nonsense suppressor, the ß-39 mRNA levels were restored to normal. This suggests an unexpected communication between events in the cytoplasm such as translation and intranuclear metabolism. The potential existence of this communication is supported by the experiments described in this dissertation.

The work by Takeshita et al. (1984) has raised some interesting questions. For example,

at what level is ß-39 mRNA metabolism affected by suppression of the nonsense mutation? My experiments as well as those of Takeshita et al. (1984) suggest that suppression of the nonsense mutation in the cytoplasm affects intranuclear events. Post-suppression experiments designed to look at transcription initiation rate (nuclear run-offs), nuclear mRNA steady-state levels and nuclear mRNA stability would attempt to address some of the possibilities. Furthermore, it is also necessary to show that the nonsense mutation in the ß globin mRNA is actually being suppressed and that the full-length protein is being synthesized. This requires the use of anti-ß globin antibodies which are not readily available to us.

Because nonsense mutation suppression could be used as a way to dissect the mechanism of the effect of nonsense mutations on mRNA metabolism, I decided to repeat and extend the experiments of Takeshita et al. (1984). However, after repeated attempts, I was never able to see an effect on ß-39 mRNA levels by co-transfection of the suppressor tRNA gene with the ß-39 gene. The exact reason for this is unknown. The possible pitfalls of this experiment include 1) low transfection efficiency of either the ß-nl or ß-39 genes, 2) over or under replication of the suppressor tRNA plasmid, 3) over or under expression of the suppressor tRNA, 4) lack of functional suppression or 5) incomplete aminoacylation of the suppressor tRNA. It is difficult to assay transfection efficiency directly since the standard method of expression of one of the transfected genes actually assays other parameters as well. However, a rough approximation for transfection efficiency can be obtained by examining the level of ß-nl mRNA expression. Plasmid replication can be checked by Southern blotting of Hirt lysates with a suppressor gene-specific oligonucleotide probe. For T antigen, replication and expression are antagonistic (Lebkowski et al., 1985) and this may occur for the suppressor tRNA as well. Expression of the tRNA gene can be assayed easily by Northern blot analysis using a suppressor-specific oligonucletide probe (Ho et al., 1986). Lacking ß-globin

antibodies, functional suppression of an amber nonsense could be assayed by co-transfection of a reporter gene such as thymidine kinase (Summers et al., 1983) or chloramphenicol acetyltransferase (Capone et al., 1986). The extent of aminoacylation of the suppressor tRNA can be assayed by separation of the acylated and deacylated tRNAs on acid-polyacrylamide gels followed by Northern blot analysis (Ho et al., 1986). A low efficiency of charging has occurred for a suppressor tRNA stably expresed in CHO cells which renders it nonfunctional (Ho et al., 1986). In these ways the problems with the system can be addressed.

In the successful nonsense suppression experiments that have been published, the suppressor tRNA was expressed following introduction of the DNA by infection, not transfection. Infection may be a more efficient way to achieve adequate nonsense suppression than transfection. The late defective SV40 virus bears the cloned suppressor gene and is propagated by co-infection with an early-defective SV40 virus. At the appropriate multiplicity of infection, almost all of the cells receive the suppressor gene. Unfortunately, in both the transient expression system assays in COS cells discussed in this chapter and in the stable expression system in AF8, infection cannot be used to express the suppressor tRNA gene. COS cells already express T antigen and AF8 cells are rodent cells nonpermissive for T antigen. One ß globin with a nonsense mutation, the ß-43, has been successfully expressed in HeLa cells (Atweh et al., 1988). One possible alternative is to express the ß-39 in HeLa cells either in a stable or transient state, and then to infect with the suppressor tRNA-bearing virus. Another approach is to study the expression of the ß-nl and ß-39 genes by establishment of a cell line with these genes and an inducible suppressor of nonsense mutations. Such a cell line has been established by Sedivy et al. (1987). They transfected a temperature-sensitive T antigen, a suppressor tRNA gene linked to the SV40 origin of replication, and the neomycin resistance gene into monkey kidney cells and selected for neomycin resistant colonies. At the

permissive temperature (33°C) T antigen is synthesized and rapid amplification of the suppressor tRNA occurs. At the nonpermissive temperature, T antigen is not synthesized and the cells grow normally. Propagation of the cells at the nonpermissive temperature avoids the potential lethality of a generalized suppression of amber nonsense mutations.

Chapter 4

Summary

One of the most common mutations that causes ß° thalassemia in the Mediterranean population is an amber (UAG) nonsense mutation at codon 39, the ß-39 mutation. Previous work has demonstrated that mRNAs bearing this mutation are present in decreased amounts in vivo in patients' peripheral blood and in heterologous transient transfection assays. This is surprising since this mutation would predict impaired function, rather than accumulation, of the ß globin mRNA. I have investigated the mechanism of decreased mRNA accumulation in two ways.

The aim of the first set of experiments was to investigate the mechanism which reduces accumulation of the human ß-39 mRNA when compared to ß-nl in mammalian cells. It had been suggested from studies in transient expression systems that the defect in expression of the ß-39 mRNA was likely to be intranuclear (Takeshita et al, 1984, Humphries et al., 1984). The results presented here have confirmed and extended their work. In order to have a constant source of mRNA, AF8 cell lines which constitutively express either the ß-39 mRNA or the ß-nl mRNA were constructed. The phenomenon of decreased ß-39 mRNA accumulation when compared to ß-nl was reproduced in these stable cell lines for total, cytoplasmic and nuclear ß mRNA. I have applied the techniques of molecular biology to study these well-characterized cell lines in order to focus on the point in the pathway for gene expression at which the defect in expression occurs. I investigated whether there was a defect in 1) the transcription initiation rate, 2) the accuracy of transcription initiation and mRNA splicing, 3) polyadenylation or 4) the mRNA half-life. I found that 1) transcription initiation frequencies of the ß-39 and the ß-nl mRNA's are approximately equal, 2) ß-39 and ß-nl mRNA's are correctly initiated, 3) ß-39 and ß-nl mRNA's

are correctly spliced, 4) ß-39 and ß-nl mRNA's are polyadenylated, 4) ß-39 and ß-nl mRNA's are stable for up to 50 hours after shift-up to the non-permissive temperature in AF8 cells, and 5) ß-39 and ß-nl mRNA's are stable for at least 3 hours in actinomycin D. I have investigated and eliminated a number of possible explanations for the defect in expression of the ß-39 mRNA . However, I was not able to investigate 2 remaining control points for gene expression: intranuclear mRNA stability and nuclear-cytoplasmic mRNA transport. Based on the data that I have generated, the hypothesis that I favor to explain the reduced accumulation of the ß-39 mRNA is as follows. Either there is a primary defect in nuclear-cytoplasmic transport of the ß-39 mRNA with a secondary defect in intranuclear stability or there is a primary defect in intranuclear stability of the ß-39 mRNA.

In my second approach I investigated whether the phenomenon of decreased mRNA accumulation of ß-39 mRNAs would be seen with other nonsense mutations within the ß globin gene. I have studied the effect of several nonsense and missense mutations in the human ß globin gene on mRNA metabolism. I constructed amber (UAG) mutations at codons 17 and 82, an opal (UGA) mutation at codon 37 and an ochre (UAA) mutation at codon 39. In addition I have corrected the UAG at the ß-39 gene to two missense mutations AAG and GAG. The mutations at ß-17, ß-37, and ß-39(UAG) have been described in patients with ß° thalassemia. This series of mutants allowed me to ask 1) if nonsense mutations at other positions in the gene would express mRNA in reduced amounts, 2) if the ochre (UAA) mutation at ß-39 would have the same phenotype as the amber (UAG) mutation at this codon, and 3) if the missense mutations at ß-39 would have an effect. Assays of steady-state mRNA levels were performed by S1 nuclease analysis of mRNA from Cos-7 cells transfected with the various mutants. I have found that the ß globin mRNA's transcribed from genes with nonsense mutations accumulate to reduced levels while the genes "corrected" with missense mutations at codon 39 make normal levels of ß globin

mRNA. This suggests that in the region of the gene we have studied the phenomenon of reduced mRNA accumulation is a general property of nonsense mutations.

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