

Targeting of a Human Iron–Sulfur Cluster Assembly Enzyme, *nifs*, to Different Subcellular Compartments Is Regulated through Alternative AUG Utilization

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Summary

Iron–sulfur clusters are prosthetic groups that are required for the function of numerous enzymes in the cell, including enzymes important in respiration, photosynthesis, and nitrogen fixation. Here we report cloning of the human homolog of NifS, a cysteine desulfurase that is proposed to supply the inorganic sulfur in iron–sulfur clusters. In human cells, different forms of NifS that localize either to mitochondria or to the cytosol and nucleus are synthesized from a single transcript through initiation at alternative in-frame AUGs, and initiation site selection varies according to the pH of the medium or cytosol. Thus, a novel form of translational regulation permits rapid redistribution of NifS proteins into different compartments of the cell in response to changes in metabolic status.

Introduction

Iron–sulfur proteins are defined as proteins in which the iron bound to the protein is at least partially coordinated by sulfur. In many of these proteins, the iron is bound to inorganic sulfur in a metallocluster, and the cluster is frequently ligated to cysteines in the polypeptide. In these proteins, which are found in a wide range of organisms from bacteria to man, the iron–sulfur cluster most commonly functions to facilitate electron transfer and less frequently contributes to catalytic function or to the maintenance of structural integrity (reviewed in Lippard and Berg, 1994; Beinert et al., 1997). Many iron–sulfur proteins are found in the mitochondria of mammalian cells, and within the cytosol there is one known iron–sulfur protein, iron regulatory protein 1 (IRP1), a regulatory protein important in the posttranscriptional regulation of genes of iron metabolism (Hentze and Kuhn, 1996; Rouault and Klausner, 1996a, 1996b).

Until recently, little was known about the mechanisms of iron–sulfur cluster assembly in cells. However, several genes important in iron–sulfur cluster assembly have now been identified in the nitrogen-fixing bacteria *Azotobacter vinelandii*, including *nifs* and *nifu* (Zheng et al., 1993). Subsequent studies have shown that NifS functions as a cysteine desulfurase, catalyzing the formation of L-alanine and elemental sulfur using L-cysteine as a substrate. NifS protein is thought to mobilize sulfur for incorporation into the metallocluster of nitrogenase (Zheng et al., 1993), and bacterial NifS catalyzes

de novo assembly of iron–sulfur clusters in vitro (Zheng and Dean, 1994). NifS homologs have been identified in numerous prokaryotes, including non-nitrogen-fixing bacteria (Zheng et al., 1993), and in the lower eukaryote *Saccharomyces cerevisiae*, where the gene was originally identified as a suppressor of a nuclear tRNA splicing defect (Kolman and Söll, 1993). The existence of NifS homologs in a wide variety of species is consistent with the possibility that NifS activates the sulfur utilized in synthesis of a variety of iron–sulfur clusters and perhaps for other types of biochemical modifications that require sulfur.

In IRP1, an iron–sulfur cluster plays a critical role in determining regulatory function. When cells are iron replete, a [4Fe-4S] cluster is assembled, and IRP1 functions as a cytosolic aconitase (Haile et al., 1992; Kennedy et al., 1992). When the iron–sulfur cluster is absent, IRP1 binds with high affinity to RNA stem loops in target transcripts known as iron-responsive elements (IREs), decreasing the translation rates of some transcripts such as ferritin or the turnover rates of other transcripts such as the transferrin receptor (reviewed in Hentze and Kuhn, 1996; Rouault and Klausner, 1996b). Thus, to fully understand the regulation of IRP1, it is important to understand the mechanisms of assembly and disassembly of iron–sulfur clusters.

Using sequence homology to bacterial NifS, we have cloned the human homolog of bacterial NifS and characterized the expression and subcellular localization of its gene product. We show that a single transcript of the human *nifs* homolog encodes proteins that are targeted to different subcellular localizations, based on initiation of synthesis at alternative in-frame AUGs. Furthermore, the relative utilization of the in-frame AUGs differs depending on the metabolic state of the cell, and a novel form of translational regulation is described.

Results

Cloning of the Human *nifs* Homolog

The human EST database was searched using the sequences for bacterial NifS protein, and a human cDNA that was close in predicted protein sequence to bacterial NifS was identified. To complete the 5' end of the transcript, experiments using 5' RACE and CapFinder were performed (Figure 1), additional 5' sequences obtained by RACE and CapFinder were sequenced, and a complete cDNA was assembled through cloning. The 5' region was exceptionally difficult to amplify, and sequencing revealed that the region was highly GC rich and had the potential to assume a stable secondary structure.

Sequence comparisons showed that the human NifS homolog was 37% identical to the bacterial NifS and 57% similar. Both a lysine residue critical in binding of the pyridoxal 5-phosphate required for enzymatic function and a cysteine required for cysteine desulfurase activity were conserved in the human sequence (Zheng et al., 1993), and lysates of COS cells transfected with human NifS supported assembly of the iron–sulfur cluster of IRP1 (data not shown). The role of NifS in facilitating iron–sulfur cluster assembly will be the subject of a

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Figure 1. 5' Nucleotide Sequence of Human *nifs* Homolog cDNA
The 5' sequence obtained by 5' RACE and CapFinder is shown. Note that an extra G nucleotide is present in the 5' end of the human *nifs* cDNA sequence obtained by CapFinder, which is not present in the corresponding genomic sequence, indicating that the 5' end of the cDNA is complete. A nonconservative TATA-like element of the promoter 23–28 nucleotides 5' of the transcription start site is underlined. The positions of AUGs 1–3 are marked above the nucleotide sequence.

separate report; here we focus on the expression and regulation of NifS.

In the predicted protein sequence of human NifS, an extra N-terminal extension of 60 amino acid residues is present in the open reading frame relative to the open reading frame of the bacterial NifS. When this open reading frame was analyzed with the P-sort program used to predict subcellular localization (Nakai and Kanehisa, 1992), an N-terminal amphipathic positively charged α helix was identified as a putative mitochondrial targeting sequence (Figure 1). A clear alignment between the bacterial protein sequence and the open reading frame distal to the second AUG codon in the sequence was identified (Figure 1).

Overexpression of NifS Demonstrates that a Single Transcript of Human *nifs* Can Encode Mitochondrial and Cytosolic/Nuclear Forms of Protein

Since iron–sulfur clusters are present in proteins in mammalian mitochondria, including citric acid cycle proteins and respiratory proteins, and in the mammalian cytosol where IRP1 is found, enzymes involved in the assembly of iron–sulfur clusters are expected in at least two distinct subcellular locations. Accordingly, we examined the expression and localization by immunofluorescence microscopy of NifS in COS cells transfected with an expression construct that included the entire open reading frame and the complete 5' portion of the transcript as identified in Figure 1. Almost all of the NifS protein was detected in mitochondria (Figure 2A), as judged by staining with the mitochondrial marker rhodamine 123 (Figure 2B), and double-labeling studies with rhodamine and NifS antibody confirmed that the two could be superimposed. However, in some cells, staining could be detected in mitochondria, cytosol, and nucleus (not shown), indicating that a single transcript

could encode differentially targeted proteins. Because the predicted product of translation initiating at the second in-frame AUG aligned well with the bacterial form, we considered the possibility that recombinant NifS in the cytosol and nucleus was the product of initiation from AUG2. Accordingly, a second construct in which the sequence 5' of the second AUG codon was removed was also used to transfect COS cells, and in these transfections, NifS protein could be detected predominantly in the cytosol and nucleus (Figure 2C).

Metabolic Labeling Confirms that Translation Is Initiated at Three In-Frame AUGs within a Single *nifs* Transcript

The predominant expression product in COS cells transfected with the full-length cDNA was a 47 kDa protein, but in addition, a 44 kDa protein was identified by immunoprecipitation of the epitope-tagged products after 1 hr metabolic labeling, and a less prominent 49 kDa product was also observed (Figure 3B, lane 3). A construct in which the native sequence 5' of AUG2 was removed was also transfected, and the major transfection-dependent band comigrated with the 44 kDa band expressed from the full-length construct (Figure 3B, lane 2). Pulse–chase experiments verified that the 49 kDa translation product was rapidly processed into a shorter cleavage product of approximately 47 kDa (quantitation reveals that the loss of counts in the precursor band at the 15 min time point of Figure 3C is accounted for by an equal increase in counts in the mature mitochondrial protein), a pattern typical of mitochondrial matrix proteins, which usually undergo proteolytic removal of the leader sequence in the final stage of protein import (Komiya and Mihara, 1996). The two shorter protein variants identified after 5 min of labeling (Figure 3C) were not subject to post-translational processing and were not derived from processing of the larger molecular weight translation product. In Figure 3B, appearance of a band larger than 49 kDa and a band smaller than 44 kDa may be due to coassembly with other iron–sulfur cluster assembly proteins.

To unequivocally verify that NifS proteins identified in COS cell overexpression experiments were the products of initiation at different AUG codons in transfected COS cells, mutagenesis experiments were performed in which each of the first two in-frame AUG codons in the full-length transcript was individually replaced with a UUG codon, and recombinant NifS proteins derived from these constructs were identified by immunoprecipitation. Substitution of the first AUG codon by a UUG codon resulted in a 10-fold decrease in expression of the 47 kDa protein, but translation from the second AUG was doubled (Figure 3D, lane 2). Substitution of the second AUG codon resulted in the disappearance of the major detectable shorter form of the protein (Figure 3D, lane 3), supporting the conclusion that the shorter form detected in cells transfected with full-length wild-type construct represented the product of initiation at the second in-frame AUG codon. Similar results were also obtained reproducibly using an *in vitro* translation system (Figure 3E). The shorter translation product(s) produced both in transfected COS cells and in *in vitro* translation reactions

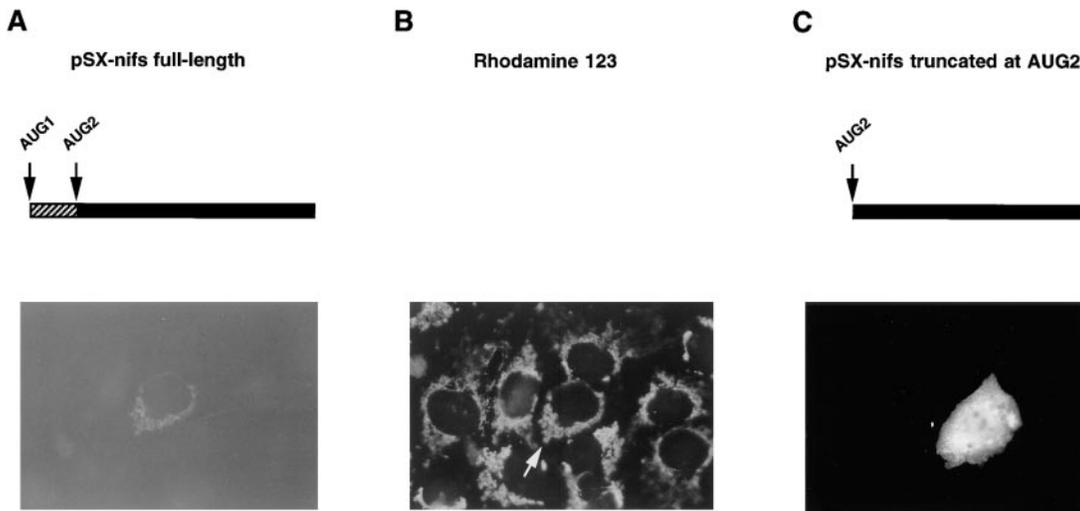


Figure 2. Immunolocalization of Epitope-Tagged Human NifS Proteins in Transfected COS Cells

COS cells transfected with myc-tagged *nifs* plasmids are stained with 9E10 followed by fluorescein-conjugated donkey antibodies to mouse IgG.

(A) NifS is localized to mitochondria in cells transfected with the full-length construct.

(B) Mitochondrial staining of cells transfected with the full-length construct by rhodamine 123. The transfected cell shown in (A) is indicated with arrow.

(C) NifS is localized to cytosol and nucleus in cells transfected with the construct that lacks sequence 5' of the second AUG.

migrated as a doublet on SDS-PAGE. To assess whether the lower band of the doublet was a processed form of one of the upper bands or was translated independently from a third in-frame AUG located 17 codons downstream from the second AUG (see Figure 1), we analyzed in vitro translation products from a construct in which the third AUG was replaced by the sequence UUG. Mutagenesis of the third AUG resulted in the disappearance of the lower band of the doublet (Figure 3E, lane 4), indicating that the third AUG was also used independently to initiate translation in vitro and was the probable source of the lower band of the doublet observed in many COS cell transfections. For unknown reasons, mutagenesis of AUG2 decreased initiation at AUG3 and increased utilization of AUG4 in the in vitro translation system. A translation product resulting from initiation at AUG4 (located 42 codons downstream from the second AUG) has not been observed in COS cell transfections, and this observation may have little relevance to in vivo expression. The products of in vitro translation were most useful in assigning the origins of the various NifS bands; the uppermost band comigrated with the short-lived precursor of the mitochondrial form observed in transfections, as expected, since processing of mitochondrial proteins would not occur in a reticulocyte lysate, and the characteristic product that resulted from initiation at AUG3 was identified by mutagenesis.

To assess the role of the initiation context and leaky scanning in expression from downstream AUGs, the nucleotide sequence surrounding AUG1 was mutagenized from ACCAUGCUG to ACCAUGGUG, an optimal context for initiation in eukaryotic cells (Kozak, 1989). Changing the context around the first AUG to an optimal context decreased but did not eliminate initiation at AUGs 2 and 3 (data not shown).

A Single *nifs* Transcript and Gene Are Detected in Northern and Southern Blots

Northern blot analyses indicated that a single detectable *nifs* transcript of approximately 2.4 kb was found in K562 cells and human heart (Figure 4A, lanes 1 and 2). To determine whether multiple genomic *nifs* sequences were present in the human genome, Southern blot analysis was performed using a 118 bp probe derived from exon III, and in each case, a single fragment was detected after probing at low stringency (Figure 4B), indicating that there are no other genomic sequences with high homology to the identified genomic *nifs* sequence, and that all cellular NifS is likely expressed from a single gene.

We considered the possibility that alternative splicing or alternative initiation of transcription could result in deletion of the first AUG codon and thereby allow differential targeting of endogenously expressed NifS without producing a detectable change in the size of the transcript. To assess more carefully potential heterogeneity of the 5' end of the transcript, we performed RNase H cleavage followed by Northern blot analysis of the transcript. An oligonucleotide corresponding to the positions 406–426 of the transcript was hybridized to the poly A⁺ RNA from K562 cells and human heart, and the hybridized RNA–DNA duplex was cleaved with RNase H. A nondigested 5' mRNA fragment was analyzed by Northern blotting, and a single product of 405 bp was detected in both K562 cells and human heart (Figure 4A).

To verify that a single *nifs* transcript is also expressed in transfected COS cells, we performed RNase H–Northern blot analysis on mRNA from the COS cells transfected with the full-length construct, and we identified a single transcript about 250 nucleotides longer than the endogenous transcript that was present (Figure 4A,

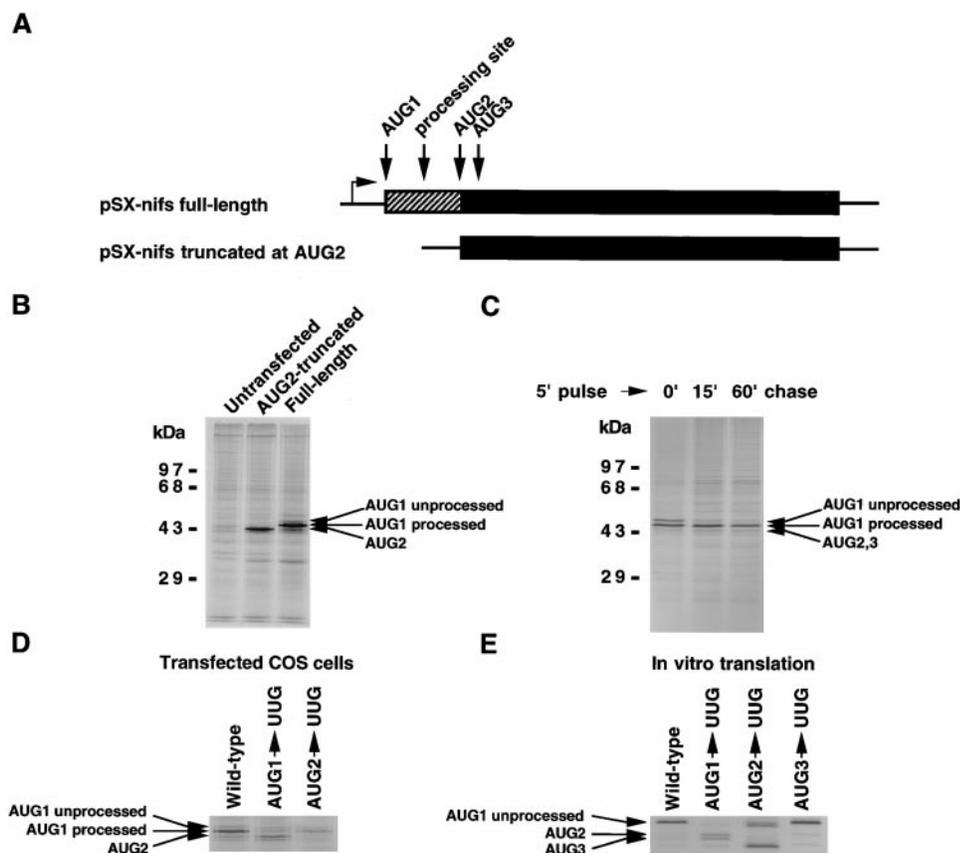


Figure 3. Multiple NifS Products Are Present in Transiently Transfected COS Cells

(A) Schematic diagram of the *nifs* locus and constructs used. The 5'-untranslated region was included in the pSX-*nifs* full-length construct. (B) NifS proteins were immunoprecipitated with 9E10 antibodies from lysates of COS cells transiently transfected with pSX-*nifs* truncated or pSX-*nifs* full-length constructs bearing a myc epitope tag in the carboxy terminus of proteins. Cells were metabolically labeled for 1 hr prior to harvesting. (C) Pulse-chase of COS cells transiently transfected with pSX-*nifs* full-length construct. Cells were metabolically labeled for 5 min followed by washing and growing with normal medium for 0, 15, and 60 min, respectively. NifS proteins were immunoprecipitated with 9E10 antibodies. (D) Removal of AUG1 results in enhanced expression of NifS from AUG2. NifS proteins were immunoprecipitated with 9E10 antibodies after 1 hr metabolic labeling. The proteins encoded by AUGs 1 and 2 are identifiably absent when either AUG 1 or 2 is mutagenized. (E) In vitro translated NifS proteins were immunoprecipitated with 9E10 antibodies. Mutagenesis of AUG#1 results in preferential utilization of AUGs 2 and 3 for initiation. Mutagenesis of AUG#3 results in the specific loss of one band, whereas mutagenesis of AUG#2 results in loss of the two lower NifS bands.

lane 5), as expected, since SR α -containing expression vectors encode extra sequence 5' of the polycloning site (Takebe et al., 1988).

A genomic fragment that contained the 5' end of the *nifs* transcript was cloned, and mapping of intron-exon boundaries revealed that an intron was present 5' of the second AUG codon, but no other intron-exon boundaries were detectable further 5' within the transcript (Figure 1). The 5' cap site was identified unequivocally through use of the CapFinder, a technique in which the 5' primer for PCR is designed to bind to 7-methyl guanosine caps. Although several different clones obtained with the CapFinder contained guanosine nucleotides at their 5' end, the true endogenous cap site could be identified because the cloned product contained an extra G that was not present in the corresponding genomic sequence and was included because the guanosine cap of the mRNA was amplified in the PCR reaction. Sequences from all of these clones aligned completely

with the cDNA sequence (Figure 1). In the genomic sequence, the region 23–28 nucleotides 5' of the identified transcription start site contained a sequence in which 5 of 6 nucleotides were T's or A's, consistent with the possibility that this region was a TATA-like element of the promoter. Primer extension and S1 analysis were unsuccessful in identifying the cap site, possibly because the 5' end of the transcript could not be quantitatively unfolded in these experiments.

Human *nifs* mRNA is predominantly found in heart and skeletal muscle, and lower mRNA levels are present in brain, liver, and pancreas (Figure 4C).

Characterization of Endogenous NifS Proteins

To determine whether endogenous NifS proteins are targeted to different subcellular locations, antibodies raised against peptides within the predicted open reading frame of *nifs* were used to analyze fractionated cell lysates. Approximately equal amounts of 44 and 47 kDa

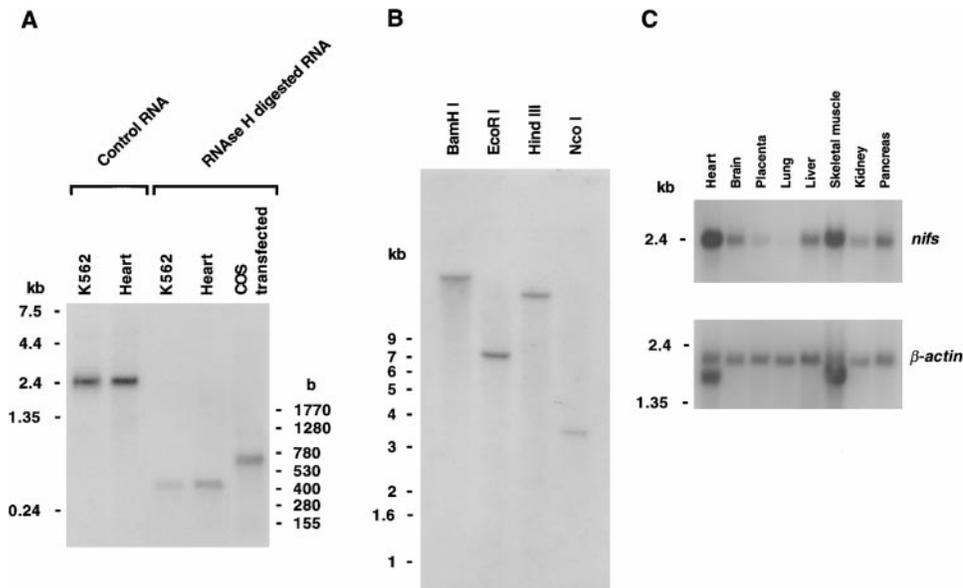


Figure 4. A 118 bp Probe Corresponding to Exon III of Human *nifs* Homolog Detects a Single Transcript and a Single Gene
(A) Northern blot of human heart, K562, and transfected COS poly A⁺ RNA. RNA was hybridized to an oligonucleotide corresponding to the positions 406–426 of the transcript, followed by RNase H digestion and Northern blotting. A single 405 bp RNase H protected fragment is obtained.
(B) Southern blot of human genomic DNA. Human genomic DNA samples (10 μg) were digested with BamHI, EcoRI, HindIII, and NcoI, electrophoresed, blotted, and probed with an exon III probe.
(C) Human multiple tissue Northern blot (Clontech) reveals that *nifs* is expressed in various tissues, with the highest levels detected in heart and skeletal muscle. Human β-actin cDNA probe was used as a control. In heart and skeletal muscle, there are two forms of β-actin mRNA, a 2 kb one and a slightly smaller one (Giovanna et al., 1991).

proteins were detected in human K562 cell lysates (Figure 5A, lane 1), and subcellular fractionation revealed that the 44 kDa protein was found in the cytosol and nucleus, whereas the longer form was found exclusively in mitochondria (Figure 5A, lanes 2–4). A putative nuclear localization motif identified in P-sort with the sequence RRPR at position 271 of the protein may be important for the nuclear localization of some of the protein.

The discovery that equal amounts of mitochondrial versus cytosolic and nuclear NifS were present in K562 cells led us to characterize the ratio of long/short NifS proteins in several other cell types and tissues. In renal oncocytoma cells, which are characterized by high respiration rates and high numbers of mitochondria (Sybren-Meijer et al., 1982; Choi et al., 1983), predominantly mitochondrial protein was detected by Western blotting, whereas in renal clear cell carcinoma cells, which have fewer mitochondria, both NifS proteins were found (Figure 5B).

To determine whether the differing ratios in long to short forms were attributable to differences in the rate of synthesis, metabolic labeling studies and immunoprecipitations were performed in cell lines. In K562 cells, the distribution between mitochondrial and cytosolic/nuclear forms was equal (Figure 5B, lane 1), and the rates of synthesis of the products of initiation at AUGs 1 and 2, identified by comigration of these proteins with overexpressed proteins on SDS-PAGE gel, were also equal. (Figure 5C). The characteristic product of initiation at AUG3 was also detected below the AUG2 product, and utilization of AUGs 2 and 3 to initiate translation

in K562 cells was much higher than was seen in COS cells transfected with recombinant *nifs*. The approximately 44 kDa band seen on Western blotting in K562 cells (Figure 5B, lane 1) was close in size to the products of both AUG2 and 3 that were distinguished in metabolic labeling experiments (Figure 5C, lane 3), and it is not clear why only two forms of protein were resolved on Western blotting.

Regulation of the Differential Utilization of In-Frame AUGs of the *nifs* Transcript

Since there is significant variation in the amounts of short and long protein in various cell types, we considered the possibility that cells may vary the amounts synthesized from each initiation codon according to the needs of individual cell types. Metabolic labeling experiments showed that the biosynthetic ratio of long/short NifS proteins was 10:1 in oncocytoma cells after 15 min of labeling, whereas that ratio was 3:1 in renal clear-cell carcinoma cells (data not shown), showing that the relative utilization of the in-frame AUGs differed in these two distinct renal carcinoma cell types.

Repeated determinations of the ratios of newly synthesized forms of NifS in K562 cells showed considerable variation depending on cell culture conditions, indicating that individual cells may vary AUG utilization in accordance with changes in metabolic status. The biosynthetic ratios of forms of NifS proteins from K562 cells grown in media with different pH were compared, and lowering the pH over the physiological range 7.3–6.8 caused a decrease in the amount of cytosolic/nuclear

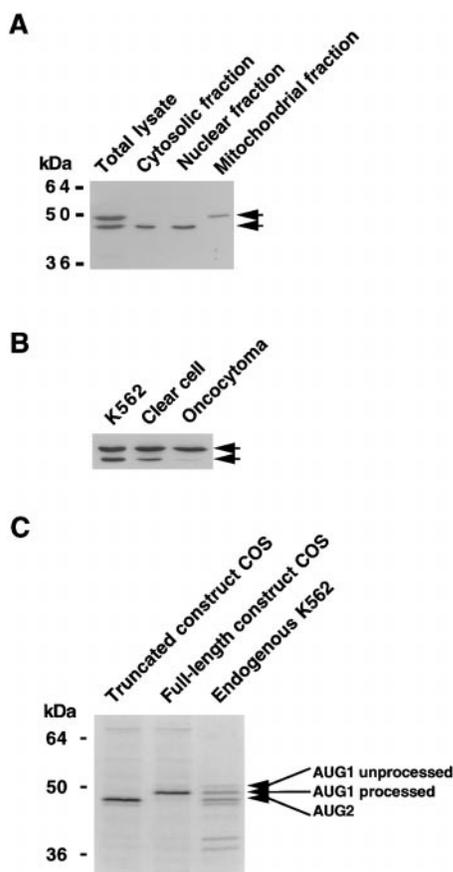


Figure 5. Endogenous Forms of NifS Proteins Are Synthesized Independently from In-Frame AUGs and Localize to Three Subcellular Locations

(A) Two endogenous NifS proteins with different subcellular localizations are equally well recognized by the polyclonal antibody. K562 cells were fractionated as described in Experimental Procedures. Fractions were analyzed by Western blotting using affinity-purified polyclonal anti-peptide antibody 2755. NifS proteins are indicated with arrows.

(B) Relative amounts of the mitochondrial versus cytosolic/nuclear form differ in cell lines. The steady state distribution in K562 cells, in renal clear cell carcinoma, and in renal oncocytoma, a cell type that is extremely rich in mitochondria.

(C) Immunoprecipitation of endogenous NifS proteins reveals that two in-frame AUGs are synthesized independently with 1:1 biosynthetic ratio. Immunoprecipitates of NifS from COS cells transfected with the construct that leads to synthesis of the mitochondrial form or the cytosolic/nuclear form are shown to indicate the positions of the proteins on the gel. Immunoprecipitates (described in Experimental Procedures) from metabolically labeled K562 cells indicate that the rate of synthesis from AUG2 is equal to that from AUG1.

NifS protein along with a relative increase of the amount of mitochondrial NifS synthesized. The biosynthetic ratio of long/short NifS proteins in K562 cells was 1:1 when the pH of the medium was 7.3, whereas the proportion of the long, mitochondrial NifS protein gradually increased, and the proportion of the short, cytosolic/nuclear NifS protein simultaneously decreased when the medium pH was lowered (Figure 6A). The overall rate of translation dropped 30% at pH 6.8 as judged by total counts incorporated, and the decrease in total NifS synthesis was proportional.

To confirm that intracellular pH can regulate the relative utilization of in-frame AUGs of the *nifs* transcript, K562 cells were treated with ethylisopropylamiloride (EIPA), a specific inhibitor of the Na^+/H^+ exchanger that regulates intracellular pH (Ingber et al., 1990). When K562 cells were treated with 40 μM of EIPA for 24 hr, the biosynthetic ratio of long/short NifS proteins increased to 4:1 (data not shown), showing that the differential utilization of the in-frame AUGs can be induced by changes in intracellular pH without changes in the pH of the media.

To characterize the time-course required for pH regulation of AUG utilization in *nifs*, K562 cells were grown at pH 6.8 for 24 hr, and after a switch to growth in media at pH 7.4, cells were harvested at several time points to evaluate the time course over which the change in AUG utilization occurs. Increase in the pH of the medium to 7.4 caused a gradual decrease of synthesis of the mitochondrial form over the 6 hr time course (Figure 6B), along with a compensatory increase in the cytosolic/nuclear forms. Equal amounts of labeled proteins were used in immunoprecipitation, and quantitation of the NifS proteins showed that the total amounts of the labeled long plus short NifS proteins remain constant, indicating that the increase in pH resulted in the coordinate decrease of translational initiation from the first AUG and increase from the downstream AUGs. At pH 6.8, the processing of the mitochondrial NifS protein was inhibited (Figure 6B, lane 1), consistent with the observation that mitochondrial protein import depends on maintenance of a proton gradient across the mitochondrial membrane (Huckriede et al., 1996).

Discussion

Targeting of NifS to Different Subcellular Compartments of the Cell Is Accomplished by Initiation of Translation from Alternative In-Frame AUGs

In mammalian cells, iron-sulfur proteins are found both in the mitochondria and the cytosol, and therefore, cells must possess targeting mechanisms that ensure that enzymes critical in iron-sulfur cluster assembly can be localized to the appropriate subcellular compartments. Differential targeting can potentially be achieved by several distinct mechanisms, including expression and regulation of two or more separate genes, or, when a single gene is involved, by alternative initiation of transcription, alternative splicing with removal of an in-frame AUG from the transcript, or alternative translational initiation. The identification of a single homogeneous NifS transcript with a single cap site permits us to rule out a role for alternative transcription or splicing in NifS expression, and Southern analysis shows no evidence for a second gene. Pulse-chase studies verify that initiation occurs at several in-frame AUGs, and targeting to mitochondria occurs only in NifS that is the product of initiation at AUG1. NifS that results from initiation at AUG2 or 3 localizes to the nucleus or cytosol, and the factors that determine the distribution between these compartments are not yet known.

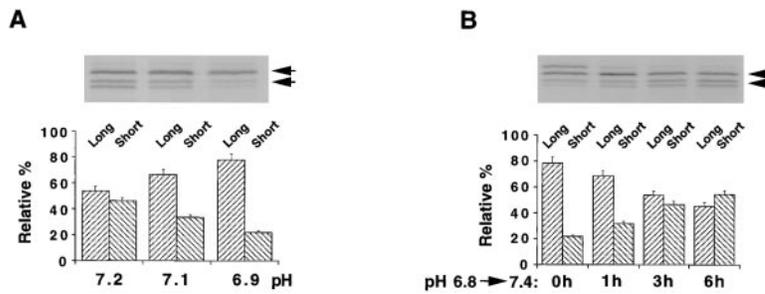


Figure 6. Relative Utilization of In-Frame AUGs of the *nifs* Transcript Is Regulated by Changes in pH

(A) K562 cells grown at the indicated pH for 48 hr were metabolically labeled for 15 min and the lysates were immunoprecipitated. Quantification of the long/short protein ratio was performed with a Molecular Dynamics Phosphorimager. (B) The ratio of mitochondrial to cytosolic/nuclear NifS protein synthesis alters within hours according to pH. K562 cells were grown at pH 6.8 for 24 hr, followed by the increase of pH to 7.4 for 1 hr, 3 hr, and 6 hr, respectively, before metabolic labeling for 15 min. Total amounts of labeled NifS proteins at these time points are constant.

In-Frame AUGs that Encode Different Forms of Proteins in Other Systems

In recent years, single transcripts that utilize in-frame AUGs to encode proteins that differ in localization or function have been described. Several directly analogous examples have been described for the yeast tRNA processing enzyme genes *cca1*, *mod5*, and *trm1*. Similar to the human *nifs* gene, these genes have been shown to use two in-frame AUGs for synthesizing proteins for mitochondrial and nuclear/cytosolic compartments (Martin and Hopper, 1994), although the mechanism responsible for producing more than one protein from a single gene differs among these genes. Both *cca1* and *mod5* are thought to encode proteins from different in-frame AUGs contained within full-length transcripts, but many of the transcripts do not contain the first AUG, so that both transcriptional variation and alternative translational initiation may be important. In *trm1*, different 5' ends have been identified, and AUG selection appears to be determined mainly by alternative splicing (Martin and Hopper, 1994).

Other examples of alternative AUG usage are reported in the literature, although in many cases the conclusions are based on *in vitro* translation or Western blotting rather than metabolic labeling and immunoprecipitation of endogenous proteins. These examples include rat liver fumarase gene (Suzuki et al., 1992), human ubiquitin-activating enzyme *E1* gene (Handley-Gearhart et al., 1994), mouse *LAP* gene (Descombes and Schibler, 1991), mouse *GATA-1* transcription factor gene (Calligaris et al., 1995), and the *Neurospora* clock protein *frequency* gene (Garceau et al., 1997; Liu et al., 1997).

Possible Mechanistic Explanations for Utilization of Downstream AUGs

There are several possible mechanisms that may account for the synthesis of two or more proteins from a single transcript. Selection of an initiator AUG codon in transcripts is generally thought to be determined by a scanning process that originates at the 5' 7-methyl guanosine caps and results in translational initiation from the first AUG in the transcript (Kozak, 1989). According to this model, two proteins can be synthesized from a single transcript when the sequence surrounding the first AUG is suboptimal, allowing some of the ribosomes to scan for the next AUG and initiate translation from downstream initiation codon(s) (Kozak, 1989). In

human *nifs* expression, the endogenous ratio of proteins derived from initiation at AUG1 versus initiation at AUGs 2 and 3 is 1:1 at neutral pH in several cell lines, whereas the ratio of overexpressed NifS proteins in COS cells is 20:1 (Figure 6B), and utilization of AUG1 is similarly high in RD4 cells transfected with the recombinant full-length transcript (data not shown). This difference in AUG utilization is not consistent with a simple scanning mechanism in which the initiation efficiency of translation depends solely on the nucleotide sequence surrounding the AUG codon, because marked differences in AUG utilization in experimental settings and cell types would require another variable.

A less common mechanism for translational initiation described in several viral transcripts involves cap-independent initiation from sites known as internal ribosome entry sites (Jackson et al., 1990). Evaluation of this possibility using an artificial bicistronic construct containing the EMCV IRES did not support this idea since substitution of the human *nifs* 5' sequence into the IRES site resulted in loss of expression from the second cistron (data not shown).

More recently, cap-oriented discontinuous scanning or ribosomal shunting has been described for initiation of translation at downstream open reading frames in cauliflower mosaic virus 35S RNA (Fütterer et al., 1993) and adenovirus late mRNAs (Yueh and Schneider, 1996). In this model, translation factors are proposed to associate with the mRNA cap and then translocate to a downstream AUG without scanning through the intervening sequences. RNA secondary structures of unusual stability have been described in transcripts in which shunting occurs (Fütterer et al., 1993; Yueh and Schneider, 1996). The possibility that ribosomal shunting may be involved in selection of an initiator AUG in the *nifs* transcript is supported by the observation that the 5' end of *nifs* is highly structured, as indicated by difficulties with sequencing of the cDNA, unusual difficulties with cloning the 5'UTR through PCR, and from the stability of the computer-predicted secondary structure of mRNA (Zuker, 1989). Notably, the AUGs are found near the ends of stem loops, a finding that may be important in function.

In regard to a possible shunting mechanism, the relative utilization of the in-frame AUGs in recombinant transcripts expressed in COS cells is of interest. Expression from recombinant *nifsS* constructs differs from endogenous expression in that initiation occurs predominantly

at the first AUG (Figure 5C, lane 2) whereas initiation at AUGs 2 and 3 occurs frequently in some endogenous settings (Figure 5C, lane 3). The transcript from COS cell transfections contains an extra 250 nucleotides (Figure 4A) of vector-encoded sequence 5' of the endogenous cap, a significant difference that may interfere with the ability of the transcript to allow selection of alternative in-frame AUGs and could cause translation to revert to a more normal pattern in which initiation occurs at the first AUG.

Potential Advantages of Utilization of Alternative In-Frame AUGs

While the economy of using a single gene and single transcript to synthesize distinct proteins that are targeted to different subcellular compartments may be advantageous, it is also possible that there is a regulatory advantage to closely linking the expression of two forms of a protein. In the pH-dependent regulation of NifS shown in Figure 6, an increase in synthesis of the mitochondrial form is accompanied by a proportional decrease in the cytosolic/nuclear forms. Perhaps increased synthesis of mitochondrial NifS in response to a decrease in pH is needed to increase the efficiency of respiration, possibly by enhancing mitochondrial iron-sulfur cluster synthesis, or perhaps by increasing mitochondrial tRNA biosynthesis at the expense of nuclear tRNA biosynthesis. Although the possible physiological relevance of pH regulation of biosynthesis of the long and short NifS proteins will require further study, we have observed that cytosolic/nuclear NifS proteins are rapidly degraded in K562 cells (T. L. and T. A. R., unpublished data), and thus, selective translational initiation at different AUGs is likely to be a key mechanism of coordinating *nifs* gene expression to the metabolic needs of the cell. Regulatory linkage between the expression of *nifs* in different subcellular compartments may add considerably to the potency of regulation because it may enable cells to synchronize changes in distinct metabolic compartments with precision and economy.

Experimental Procedures

Cloning of Human *nifs* Homolog

A clone containing sequence similar to bacterial *nifs* was obtained from human expressed sequence tag (EST) database. The clone was sequenced, and the missing 5' sequence was cloned by 5' RACE and CapFinder techniques (Clontech) using human heart poly A⁺ RNA (Clontech). The complete *nifs* cDNA was cloned into Bluescript vector pSK⁺ (Stratagene) and was sequenced. A human genomic DNA library was screened using the human *nifs* cDNA corresponding to the entire open reading frame as a probe. DNAs hybridized to probe were subcloned to pSK⁺ and were sequenced.

Northern Blot and Southern Blot Analysis

Poly A⁺ RNA from human heart (Clontech), K562, and COS cells was separated on a 1.5% agarose-formaldehyde gel, transferred to GeneScreen, and hybridized to human *nifs* exon III probe. Human genomic DNA was digested with the indicated restriction enzymes and separated on a 0.8% agarose gel.

Antibody Production

The rabbit polyclonal antibody 2755 was raised against synthetic multiple antigenic peptide (MAP 5) corresponding to 143–157 amino acid residues (RSRKKHLITQTQTEHK) of human NifS. Antiserum was generated in New Zealand white rabbits by Covance Laboratories

Inc. (Vienna, VA). Before use in immunoprecipitations and Western blots, the antiserum was affinity purified using the MAP 5 column.

Cell Culture, Expression Vectors, and DNA Transfection

K562 cells were grown in RPMI 1640 medium, and COS, oncocyoma, and clear cells were grown in Dulbecco's modified Eagle's medium (DMEM). All media contained 10% fetal calf serum (FCS).

Human *nifs* entire open reading frame with 5' untranslated region was subcloned into pSX, a modified version of the mammalian expression vector pCDL-SR₁ (Takebe et al., 1988), and named pSX-*nifs*. DNA constructs had a 12-amino acid epitope tag, myc (MEQKLI SEEDLN), added to the C termini of the NifS. N-terminally truncated human *nifs*-myc DNA was generated by PCR. Mutations of human *nifs*-myc DNA were generated by PCR-mediated site-directed mutagenesis.

COS cells (4–8 × 10⁶) grown in DMEM with 10% FCS were electroporated with 10 μg of DNA (4 pulses of 0.5 kV × 99 μsec; BTX Electro Square porator model T820; BTX, San Diego, CA) and incubated in DMEM/FCS for 48 hr before lysis.

Cell Lysis, Immunoprecipitation, and Western Blotting

COS, K562, oncocyoma, or clear cells were washed with ice-cold PBS and lysed in Triton X-100 lysis buffer (20 mM Tris-HCl [pH 8.0]/137 mM NaCl/1% Triton X-100/10% (vol/vol) glycerol/1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF)/0.5 μg of leupeptin per ml). Lysates were immunoprecipitated with antibody 2755 or a monoclonal anti-myc (9E10). Metabolically labeled K562 cell lysates were subjected to recapture with 2755 antibody after the immunoprecipitation. Lysates or immunoprecipitates were analyzed on SDS/10% polyacrylamide gel, and Western blots were probed with 9E10 at 1:1000 or 2755 at 1:500 dilutions.

Metabolic Labeling Experiments

Thirty-six hours after transfection, COS cells were incubated with labeling medium (DMEM without methionine and cysteine, 5% dialyzed FCS, and 0.1 mCi of Tran³⁵S-label (ICN) per ml) for 1 hr at 37°C. K562 cells were incubated with labeling medium containing 2–5 mCi of Tran³⁵S-label per ml for 1 hr at 37°C.

Subcellular Fractionation

Pellets of K562 cells were suspended in 200 μl of lysis buffer (210 mM mannitol, 70 mM sucrose, 4 mM HEPES [pH 7.2], 1 mM AEBSF, 0.5 μg of leupeptin per ml) and lysed with digitonin over 15 min. The cell homogenates were centrifuged at 600 × g for 10 min at 4°C. The pellets were washed, extracted with salt (final KCl concentration = 300 mM), and centrifuged at 15,000 × g for 30 min at 4°C, and the supernatants were named nuclear fractions. The supernatants from the first centrifugation were centrifuged at 7000 × g for 9 min at 4°C. The pellets were lysed with Triton X-100 lysis buffer and named mitochondrial fraction, and the supernatants were named cytosolic fraction.

Immunofluorescence

Transfected COS cells were fixed in 2% paraformaldehyde at room temperature for 20 min, permeabilized with methanol for 2 min, stained with 9E10 (1 μg/ml), mounted, and photographed by immunofluorescence and phase contrast microscopy as described (Humphrey et al., 1993).

In Vitro Translation

pSK-*nifs* constructs under the control of T3 promoter were in vitro translated using the TnT Coupled Reticulocyte System (Promega).

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GenBank Accession Number

The accession number for the full human *nifs* homolog cDNA sequence is AF097025.