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Internal Translational Initiation in the mRNA from the *Neurospora crassa* Albino-3 Gene*

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The "ribosome scanning model" for translational initiation predicts that eukaryotic mRNAs should, as a rule, be monocistronic. However, cases have recently been described of eukaryotic mRNAs producing more than one protein through alternative translational initiation at several different AUG codons. The present work reports the occurrence of multiple translational start sites on the mRNA of the *Neurospora crassa* gene albino-3 (al-3), encoding the carotenoid biosynthetic enzyme geranylgeranyl-pyrophosphate synthase. This was revealed by the molecular analysis of an al-3 mutant carrying a deletion within the coding sequence, which was expected to prevent the synthesis of a functional geranylgeranyl-pyrophosphate synthase because of ribosome frameshifting and premature translational termination. However, the mutants could maintain appreciable geranylgeranyl-pyrophosphate synthase activity through a mechanism operating at the translational level, whereby a fraction of ribosomes initiated protein synthesis from either of two internal in-frame AUG codons located downstream of the deletion, thus producing a shortened but still active version of the geranylgeranyl-pyrophosphate synthase. The results presented indicate that the internal AUG codons are recognized mainly or solely by direct ribosome binding rather than by "leaky scanning" from the 5′ end of the mRNA.

Initiation of translation by eukaryotic ribosomes is believed to take place by a "scanning" mechanism, whereby the small ribosomal subunit binds to the capped 5′ end of the mRNA and migrates until the initiator codon (usually the first available AUG) is reached (1). This model rationalizes many different observations, first of all the seemingly scarce ability of eukaryotic ribosomes to bind on internal sites of a mRNA, which would in turn account for the general lack of polycistronic mRNAs in eukaryotic cells.

However, in the last few years, several examples have been described of "multifunctional" eukaryotic mRNAs, where translational initiation can take place at two or more different AUGs on the same mRNA, resulting in the synthesis of proteins of different length and, often, also function (2–5). To explain these observations, a "leaky scanning" mechanism has been proposed, positing that a fraction of the scanning ribosomes would bypass the first AUG and start translation at a subsequent one (2). In a few instances, the occurrence of authentic internal initiation has been demonstrated; this may require the presence of special structural features on the mRNA molecule, although no clear consensus pattern has been evidenced (6, 7).

In this work, we describe the translational behavior of the mRNA from the *Neurospora crassa* gene albino 3 (al-3), encoding the carotenoid biosynthetic enzyme geranylgeranyl-pyrophosphate synthase. We have found that the al-3 mRNA, both *in vivo* and *in vitro*, can be decoded starting from at least 3 different in-frame AUG codons. The first is the regular initiation site for the wild-type geranylgeranyl-pyrophosphate synthase. Another two AUGs, located about 250 nucleotides downstream of the first and spaced 30 nucleotides from each other, are also recognized as translational start signals producing truncated, but still active, enzymes lacking the first 100–110 amino-terminal amino acids. The available experimental evidence indicates that initiation at the internal AUGs takes place by a mechanism of direct ribosome binding rather than by leaky scanning from the 5′ end of the mRNA.

MATERIALS AND METHODS

Strains and Culturing Media—The *N. crassa* wild-type 74-OR23A strain and the al-3 mutants rosy (FGSC 3581) and RP100 (FGSC 2082) were supplied by the Fungal Genetic Stock Center (FGSC; University of Kansas, Kansas City, Kansas). The al-3/qa-2/aro-9 (qa, quinate; aro, aromatic cluster gene) (RP100 M246 Y325M6) *N. crassa* strain had been constructed previously (8). All *Neurospora* strains were cultured according to standard procedures (9).

The *Escherichia coli* strains BW313 (*dut, ung, thi1, relA, spoT1/F′lysA*) and CSH50 (*pro-lac, ara, thi/F′ tra D36, proAB, lacI Z M15*) were used for site-directed mutagenesis experiments.

Cloning and Sequencing of the al-3 Rosy Gene—The cloning of the al-3 coding sequence was carried out as described previously (10). Sequencing was performed on both strands by the dideoxy chain termination method, using universal and specific oligonucleotide primers.

Recombinant Plasmids, Site-directed Mutagenesis, and Transformation—Plasmid pW16 was a pRAL construct carrying a 4.2-kilobase *Hin*dIII-*Nru*I *Neurospora* DNA fragment, spanning 1200 nucleotides of the al-3 promoter region, the complete al-3 open reading frame, and about 1500 nucleotides of additional downstream sequences (Fig. 1). To obtain plasmid pR16, a *Sac*I-*Sal*I fragment (comprising 640 bp[1] of the promoter region and the first 550 bp of the coding sequence) was excised from pW16 and substituted with the corresponding DNA trait derived from the rosy al-3 gene (Fig. 1).

For oligonucleotide-directed mutagenesis, *Sac*I-*Nru*I fragments derived from both pW16 and pR16 plasmids were subcloned in M13mp18. The oligonucleotide used for mutagenesis of the two 5′ proximal AUGs was 5′ CACGGCgATcGTGACGTGTTCCtTTTCCTT 3′; the mutated positions (lowercase) were chosen to create a *Pvu*I site. The oligonucleotides designed for the mutagenesis of the two distal AUGs (at positions 264 and 294) were, respectively, 5′ GTTGGGAGgATcCTGGGG 3′ and 5′ GGGCGCgATcGTGTATGG 3′; the mutations introduced created, respectively, a *Bam*HI and a *Pvu*I site. Mutant clones were obtained using the uracil selection system (11); plasmids bearing the chosen mutations were identified by restriction analysis and confirmed by sequencing.

For *Neurospora* transformation experiments, *Sac*I-*Xba*I 3.6-kilobase fragments containing the al-3 promoter and coding regions were excised from the pW16/M13 and pR16/M13, and the five different mutagenized

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[1] The abbreviation used is: bp, base pairs.

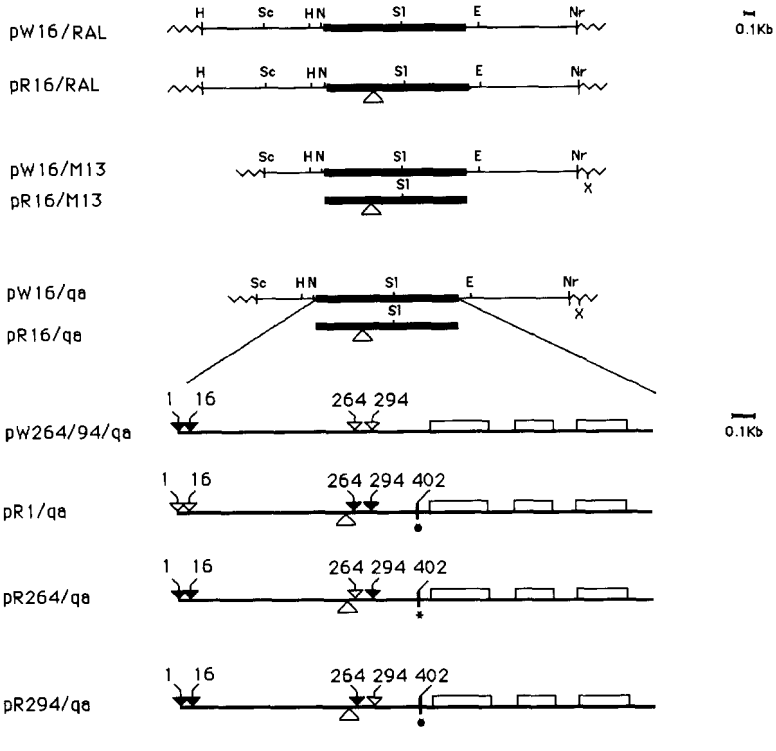

FIG. 1. **Constructs of the al-3 gene.** *pW16/RAL* and *pR16/RAL*, pRAL constructs carrying a 4.2-kilobase *Hin*dIII-*Nru*I trait of *N. crassa* DNA, containing, respectively, the wild-type and the rosy mutant gene. *pW16/M13* and *pR16/M13*, wild-type and rosy al-3 constructs used for site-directed mutagenesis. Restriction sites are indicated as follows: *H*, *Hin*dIII; *E*, *Eco*RI; *N*, *Nae*I; *Nr*, *Nru*I; *Sc*, *Sac*I; *Sl*, *Sal*I; *X*, *Xba*I. The *black box* indicates the al-3 coding sequence. *pW16/qa*, *pR16/qa*, etc., constructs used for *in vivo* expression experiments. The last five plasmids are those obtained by site-directed mutagenesis of the relevant AUG codons; the *black arrows* indicate unmodified AUGs, whereas *white arrows* indicate mutagenized AUGs. The numbers refer to the position in the coding sequence, starting from the first AUG. The *white triangle* indicates the location of the deletion; the termination codon created by the frameshift is marked with an *asterisk*. The *open boxes* represent the conserved domains shared by the prenyltransferase enzymes.

plasmids were inserted in the previously characterized pBSK/qa-9 plasmid (12) obtaining the constructs illustrated in Fig. 1. Preparation of RP100 al-3/qa-2/aro-9 spheroplasts and transformation with the recombinant plasmids were carried out according to Nelson *et al.* (8).

Recombinant plasmids for *in vitro* transcription were obtained by cloning 1.5-kilobase *Nae*I-*Eco*RI fragments (containing the untranslated leader and the coding sequence, but not the promoter), derived from, respectively, the pW16/qa, pR16/qa, pR1/qa, p264/269/qa, and pW264/269/qa constructs into a Bluescript SK vector restricted with *Kpn*I and then with *Eco*RI after filling-in of the *Kpn*I site.

To investigate the mechanism of initiation at the internal AUGs, the pR16/qa/Bluescript construct was further modified by the insertion of a stem-loop structure, as follows. Two 36-mer complementary oligonucleotides (5′ AGCCATGGCCGGGCCCATATGGGCCCGGCCATGGGC 3′ and 5′ GCCCATGGCCGGGCCCATATGGGCCCGGCCATGGCT 3′) carrying *Nco*I sites at both extremities were annealed by heating at 95 °C followed by slow cooling. The duplex was digested with *Nco*I and then inserted at the *Nco*I site of the selected plasmid. After transformation, recombinant clones were identified by colony hybridization with the labeled oligonucleotides as the probe. The presence of the extra sequence in the new construct was confirmed by sequencing.

In Vitro Transcription and Translation—To obtain run-off *in vitro* transcripts, the Bluescript SK recombinants carrying the various al-3 constructs were linearized with *Eco*RI and purified by phenol extraction. Transcription with T7 polymerase and co-transcriptional RNA capping with diguanosine triphosphate were performed according to described procedures (13). The transcripts were recovered by ethanol precipitation, and their integrity and homogeneity were assessed by denaturing agarose gel electrophoresis (13).

Samples for *in vitro* translation contained, in a final volume of 25 µl, wheat germ extract (Promega), 12.5 µl; RNase inhibitor (33.6 units/ml), 1 µl; amino acids minus methionine, 80 µM; potassium acetate, 50 mM; [^{35}S]methionine (specific activity, 1200 Ci/mmol), 0.5 mCi/ml; RNA transcripts, 1–2 µg. The reaction mixtures were incubated at 25 °C for 60 min. To analyze the products of translation, aliquots (5 µl) of the samples were electrophoresed for 3 h at 100 V on 15% SDS-acrylamide gels. At the end of the run, the gels were dried and autoradiographed.

RESULTS

Nature of the al-3 Rosy Mutation—The *N. crassa* al-3 gene encodes geranylgeranyl-pyrophosphate synthase, a 47-kDa prenyltransferase enzyme indispensable for the biosynthesis of carotenoids (8, 10, 14). Amino acid sequence comparison has shown that geranylgeranyl-pyrophosphate synthase shares with other prenyltransferases three highly conserved regions, which probably define the functionally important domains of the enzyme (15).

The present work started with the sequencing of a mutant al-3 gene (termed rosy), which determines a pale orange phenotype due to a reduced synthesis of carotenoids. The mutation turned out to be a 13-nucleotide deletion located 243 bp from the opening AUG of the al-3 reading frame (Fig. 2). The frameshift provoked by the deletion creates a termination codon 402 bp from the initiator AUG, thus defining a truncated polypeptide lacking all of the prenyltransferase consensus regions (Fig. 2). As al-3 is a single-copy gene (8), the rosy mutants would be expected to be fully devoid of active geranylgeranyl-pyrophosphate synthase.

However, these mutants are still able to synthesize substantial amounts of carotenoids and must, therefore, possess appreciable geranylgeranyl-pyrophosphate synthase activity. In principle, such activity in the rosy strain could be restored by a concurrent frameshift-suppressor mutation or by the activation of an alternative "rescue" pathway, in which another unrelated protein would supply the missing enzymatic activity.

To test these possibilities, the pR16/RAL construct (carrying the rosy al-3 gene with its promoter region, see Fig. 1) was used to transform a *Neurospora* al-3 RP100/qa-2/aro-9 strain (see "Materials and Methods"). RP100 is another al-3 mutation (a single Ser to Asn substitution at position 386 of the protein), severely impairing geranylgeranyl-pyrophosphate synthase activity and resulting in a quasi-albino phenotype readily distinguishable from rosy (10, 14).

About half of the transformants displayed a rosy phenotype (Table I), revealing that the rosy mutation was dominant over the RP100 one. Therefore, the rosy phenotype was determined

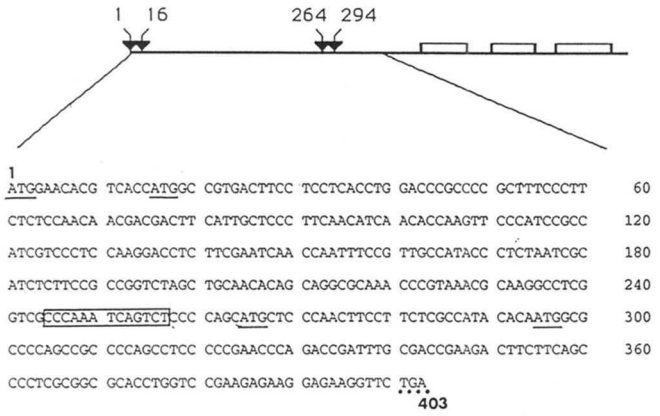

FIG. 2. **Nucleotide sequence of the 5′ half of the al-3 gene.** The upper graph shows a schematic representation of the entire al-3 coding sequence, where the *arrows* with *numbers* indicate the positions of the four relevant AUG codons. The *open boxes* represent the conserved domains shared by the prenyltransferase enzymes. The *lower part* of the figure illustrates the sequence of the first 403 nucleotides of the al-3 gene. The relevant AUG codons are *underlined*; the 13 nucleotides deleted in the rosy mutant are *boxed*; the TGA codon determined by the frameshift is indicated by a *dotted underline*.

TABLE I
In vivo expression of the different constructs realized by site-directed mutagenesis

The numbers represent the percentages of different phenotypes obtained by transformation of the *al*-3 RP100/*qa*-2 *aro*-9 *N. crassa* strain with the various *al*-3 constructs (see Fig. 1).

Constructs	Phenotypes		
	Wild-type	Rosy	RP100
pW16/RAL	46	0	54
pW264/94/qa	63	0	37
pR16/RAL	0	48	52
pR1/qa	0	44	56
pR264/qa	0	35	65
pR294/qa	0	46	54
pR264/94/qa	0	0	100

by the presence of the rosy gene itself and could not be accounted for by some kind of trans-complementation effect taking place in the rosy strain.

Site-directed Mutagenesis of the Rosy al-3 Gene—The question then arose as to how a functional geranylgeranyl-pyrophosphate synthase could still be produced in spite of the presence of the frameshift mutation.

Inspection of the gene sequence suggested a possible answer. Two AUG codons in close succession (12 nucleotides apart) are found at the beginning of the al-3 reading frame, either (or both) of which could serve as an initiation signal (Fig. 2). The predicted product is a polypeptide of 428–433 amino acids having a molecular mass of about 47 kDa. The next in-frame AUG codons are located, respectively, 264 and 294 bp from the first one and are, therefore, downstream of the rosy deletion (Fig. 2). Translational initiation at either of these internal AUGs would produce a protein lacking about 100 amino acids at its amino terminus but still containing the three intact prenyltransferase consensus regions (Fig. 2), thus possibly retaining an at least partial enzymatic activity.

To test this hypotesis, four variants of the al-3 rosy gene in which the above mentioned AUG codons were in turn modified were constructed by site-directed mutagenesis.

In plasmid pR1/qa, both of the initial AUG codons were mutated while leaving invariant the internal ones. Plasmids pR264/qa and pR294/qa retained both of the initial AUGs but lacked, respectively, either the internal AUG at position 264 or that at position 294. Plasmid pR264/294/qa bore mutations in

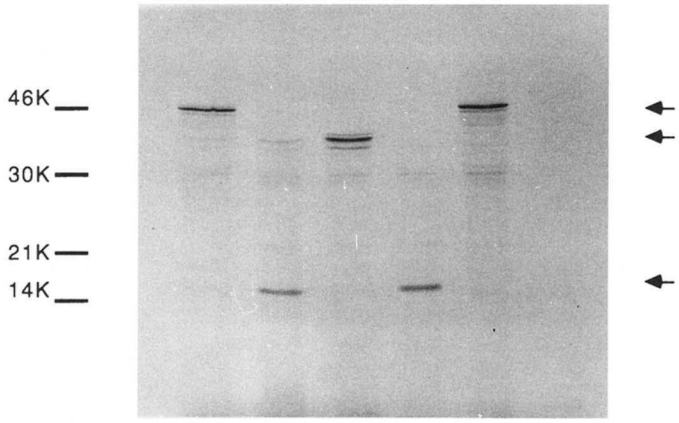

FIG. 3. ***In vitro* translation of the various al-3 constructs.** The electropherogram shows the proteins resulting from *in vitro* translation of RNA transcripts derived from the following constructs (*cf.* Fig. 1): *A*, wild-type al-3 (pW16/qa); *B*, rosy mutant (pR16/qa); *C*, rosy mutant in which the two first AUG codons were mutagenized (pR1/qa); *D*, rosy mutant in which the two internal AUG codons were mutagenized (pR264/294/qa); *E*, wild-type al-3 in which the two internal AUGs were mutagenized (pW264/294/qa); *F*, control without added mRNA. The position and size of the molecular weight markers are reported on the *left*; the *arrows* on the *right* indicate, from *top* to *bottom*, the 47, 40, and 16-kDa polypeptides. *In vitro* translation and gel electrophoresis were performed as described under "Materials and Methods."

both of the internal AUGs while keeping invariant the initial ones (see Fig. 1). As before, the constructs were used to transform the al-3 RP100/qa-2/aro-9 *Neurospora* strain, and the resulting phenotypes were analyzed.

As the results in Table I show, all of the constructs except pR264/294/qa determined the appearence of a rosy phenotype in the trasformants. Namely, the modification of the two initial AUG codons had no influence on the expression of the rosy gene, but when *both* of the internal AUGs were mutated, no exogenous geranylgeranyl-pyrophosphate synthase activity was detectable anymore. The presence of only one of the two internal AUGs was still sufficient to produce a rosy phenotype, indicating that these codons are functionally equivalent.

The possibility that the recessivity of the pR264/294/qa construct was due to inactivation of the enzyme by the double mutation was ruled out by testing a further construct, pW264/294/qa, obtained by modifying the two internal AUG codons in a wild-type al-3 gene (see Fig. 1). As shown in Table I, the transformants obtained with this plasmid had a wild-type phenotype, thus demonstrating that (i) the mutations introduced did not impair the function of the protein and that (ii) the initial AUG codon(s) of the al-3 open reading frame is (are) necessary and sufficient for a correct expression of the wild-type geranylgeranyl-pyrophosphate synthase.

In Vitro Translational Analysis of the al-3 Mutants—The above interpretation of the transformation experiments was confirmed by the analysis of the proteins produced by *in vitro* translation (in a wheat germ extract) of RNA transcripts derived from some of the previously described al-3 constructs.

The results in Fig. 3 show that, as expected, the principal product of translation of the wild-type al-3 mRNA was a 47-kDa polypeptide (*lane A*), while that of the rosy mRNA (*lane B*) was a polypeptide of ~16 kDa, corresponding to the truncated protein determined by the frameshift mutation. However, translation of the rosy mRNA also generated appreciable amounts of a 40-kDa product, having the size expected of a protein whose translation would initiate at either of the two internal AUG codons. That this was indeed the case was demonstrated by

translating the mRNAs derived from the constructs pR16/qa and pR264/294/qa (*lanes C* and *D*). The former, which lacked the initial, but not the internal, AUG codons gave as the main product the 40-kDa polypeptide, while missing entirely the 16-kDa one; the latter, which lacked the internal but not the initial AUGs, yielded no 40-kDa protein while still producing the 16-kDa one.

Notably, some amount of the 40-kDa protein was also synthesized from the wild-type al-3 mRNA (*lane A*) but disappeared when the internal AUG codons of the wild-type gene were mutated (construct pW264/294/qa, *lane E*). This indicates that a fraction of ribosomes initiated translation at the internal AUGs independent of the presence of the rosy mutation, thus excluding mechanisms associated to the presence of the frameshift such as ribosome reinitiation after the premature termination event. In addition, the perfect concordance between the *in vivo* and the *in vitro* data suggests that the peculiar behavior of the al-3 mRNA is most probably due to the inherent features of the message and not to some *Neurospora*-specific trans-acting factor.

Which Mechanism for Initiation at the Internal AUGs?—To get some insight into the mechanism of initiation at the internal AUGs, we first investigated the *in vitro* translational behavior of uncapped wild-type and rosy al-3 mRNAs. This, because the capping state of the mRNA was expected to influence the efficiency of translational initiation at the internal AUGs if these were reached by leaky ribosome scanning. The absence of the cap provoked, as expected, a marked reduction in the amount of the proteins initiated at the "true" start codon(s) (the 47 or the 16-kDa polypeptide); however, the synthesis of the 40-kDa protein was left unimpaired (results not shown).

More direct information was obtained by means of the following experiment. The Bluescript plasmid construct carrying the al-3 rosy gene (pR16/qa; see "Materials and Methods") was mutagenized by the insertion a short sequence capable, when in the RNA transcript, of folding into a very stable 10-basepair hairpin (Fig. 4, *top*). The hairpin, aimed at blocking the progress of the scanning ribosomes, was located between the two possible true initiator AUGs. In addition, the extra sequence carried two further AUG codons, so that there were now four potential initiation sites at the beginning of the al-3 open reading frame. The first two were in frame with each other and terminated at a TGA codon shortly afterwards; the third and the fourth were in frame with each other and also with the al-3 rosy message being, therefore, capable of giving rise to the 16-kDa truncated protein (Fig. 4, *top*). The combined effects of the strong RNA secondary structure and of the additional AUG codons were expected to prevent any scanning ribosomes from reaching the internal AUGs and producing the 40-kDa protein.

In vitro translation of the mRNA derived from this construct showed that, as expected, the 16-kDa truncated polypeptide was only synthesized in trace amounts; surprisingly, however, the translation of the 40-kDa protein was unimpaired or even somewhat improved (Fig. 4, *bottom*). As it is very difficult to rationalize these results in terms of any version of the scanning model, the conclusion seems almost inescapable that the internal AUG codons giving rise to the short version of the al-3 enzyme are recognized principally or exclusively by direct ribosome binding to the relevant region of the mRNA.

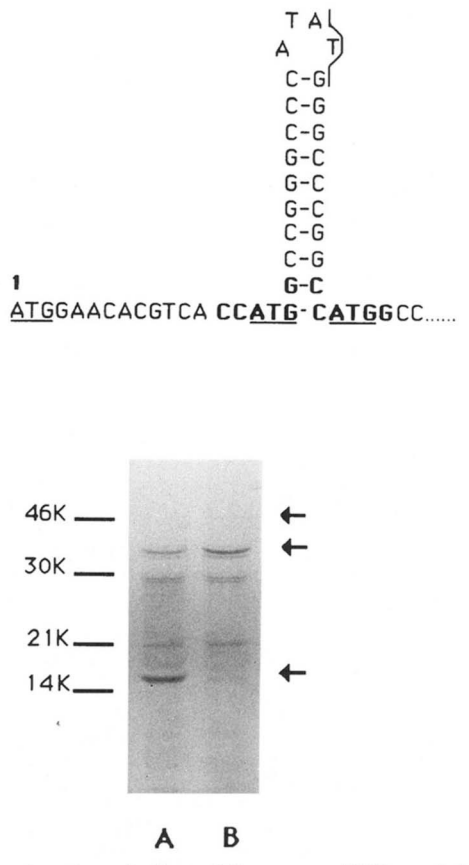

FIG. 4. ***In vitro* translation of the rosy mRNA containing the hairpin structure.** *Top*, expected folding pattern of the inserted sequence showing the putative hairpin structure and the possibile initiation codons (*underlined*); *bottom*, protein pattern resulting from *in vitro* translation of the RNA transcribed from the constructs containing the rosy al-3 gene as such (*A*) or the rosy al-3 gene modified by the insertion of the hairpin structure (*B*). The *arrows* on the *right* indicate (from *top* to *bottom*) the positions of the 47, 40, and 16-kDa polypeptides; the size markers are reported on the *left*.

DISCUSSION

The results presented in this work demonstrate that the mRNA derived from the *N. crassa* al-3 gene can be translated both *in vivo* an *in vitro* starting at several different AUG codons. Regular initiation at either of two very close AUGs produces a wild-type, 47-kDa geranylgeranyl-pyrophosphate synthase; noncanonical initiation at either of two downstream in-frame AUGs results in the synthesis of a 40-kDa protein still having enough enzymatic activity to phenotypically suppress a frameshift mutation (rosy) in the al-3 gene.

Taken together, the results suggest that "leaky ribosome scanning" from the 5′ end of the mRNA is by no means an absolute and not even a principal requirement for translational initiation at the internal AUG codons. The fact that neither a very stable upstream hairpin loop nor extra upstream AUGs can prevent the synthesis of the short al-3 enzyme indicates that this protein is most probably initiated by direct ribosome binding at, or in the vicinity of, the appropriate internal AUGs.

Internal translational initiation was first demonstrated to take place on some viral RNAs (6); more recently, instances of cellular mRNAs endowed with internal ribosome entry sites have also been reported (7). Generally, these phenomena have been interpreted as exceptions to the ribosome scanning rule, operating especially when selective competition against the normal translation mechanism is needed.

In this conceptual framework, the case of the al-3 mRNA appears *prima facie* to be anomalous, as there is no evidence that initiation at the internal AUGs is a regulated event, driven by a physiological need for the short protein. Rather, the similar efficiency of the two internal AUGs as initiation sites and the functional equivalence of the resulting proteins (which differ by 10 amino-terminal amino acids) strongly suggest the absence of any important discriminatory information in the amino-terminal portion of the full-length enzyme. Accordingly, a computer analysis failed to evidence any recognizable signal

for intracellular targeting or other known functions within the first 100 amino acids of the wild-type geranylgeranyl-pyrophosphate synthase.

The most compelling evidence that the short enzyme lacks any relevant individual function is, however, the fact that the constructs lacking the two internal AUGs (but conserving the initial ones) restored a fully viable wild-type phenotype in the RP100 transformants in spite of their inability to produce the 40-kDa protein.

Furthermore, our search for primary or secondary structural features indicating the presence of an internal ribosome entry site on the al-3 mRNA was unsuccessful (possibly because the characteristics of a *bona fide* internal ribosome entry site are very poorly defined). The only remarkable thing about the al-3 coding region is that it is exceptionally cytosine-rich (about 40% C) and guanine-poor for about one-quarter of its length, which includes the relevant internal AUGs (15). As revealed by computer analysis (data not shown), these features prevent the formation of any strong secondary structure motifs in the region of interest, while extended folding is present in the rest of the molecule. Remarkably, the unstructured region of the al-3 coding sequence contains no AUG codons (either in frame or out of frame) other than the four discussed in this work, whereas the structured remainder of the molecule contains as many as 15 AUGs in any frame (15).

Obviously, we cannot at present absolutely rule out the existence of a special ribosome entry site on the al-3 mRNA that would regulate the synthesis of the 40-kDa protein under some yet undiscovered conditions. In the light of the available evidence, however, the possibility should be considered that the internal initiation codons are directly accessible to the ribosomes for no reason other than lack of RNA folding and that the synthesis of the 40-kDa protein is the accidental byproduct of this accessibility, revealed in this particular case by the presence of an associated phenotype.

Indeed, several recent studies tend to stress the notion that mRNA structure is a primary determinant of ribosome accessibility; even the role of the cap-binding protein complex (IF-4F) is being reconsidered in the light of its ability to promote the directional unfolding of the mRNA rather than the binding of the 40 S subunits to the cap (16, 17). It is relevant in this respect that IF-4F has also been shown to promote cap-independent internal initiation (18, 19). Thus, the translational behavior of the al-3 mRNA may well be a remarkable illustration of the fact that, to quote a recent study, "... a previously recognized property of eukaryotic ribosomes should not be ignored, namely the ability to bind directly to internal open reading frames and initiate translation in a 5′ independent manner as for bacterial ribosomes" (18).

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