Enhanced Translational Utilization of Chloroplast Ribosomal Protein mRNAs from Two AUG Codons Shown by Site-Directed Mutation†

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ABSTRACT: The cDNA for protein L12 of the chloroplast ribosome contains two ATG codons, both in consensus initiator context, at the beginning of its transit peptide coding region [Giese, K., & Subramanian, A. R. (1989) *Biochemistry 28*, 3525–3529]. Due to the location in the transit peptide, translational start from either ATG codon would yield the same mature protein after transport into chloroplasts and N-terminal cleavage. To test whether this arrangement, also found in the cDNAs of several other chloroplast ribosomal proteins, could enhance the utilization of mRNA, we constructed a chimeric gene containing the 5′ part of the L12 cDNA fused to the neomycin phosphotransferase gene. A frameshift that would prematurely terminate the translation from the first AUG codon was introduced into the construct by site-directed mutagenesis. Transcription–translation of the fusion gene in vitro and its expression in vivo in spinach protoplasts showed that protein synthesis occurs from both initiation codons: at 70–80% of the total level from the first and 20–30% from the second. The results thus show that (1) eukaryotic ribosomes can initiate to a significant level from a downstream AUG codon and (2) the occurrence of two in-frame initiation codons enhances translational efficiency.

A striking feature of a recently characterized cDNA clone for the nucleus-encoded chloroplast ribosomal protein (r-protein) L12 is the presence of two identical, in-frame translational start signals at the beginning of its coding region (Giese & Subramanian, 1989). This motif of two in-frame ATG codons has also been identified in several other recently characterized r-protein cDNAs [e.g., see Phua et al. (1989), Smooker et al. (1990), and Johnson et al. (1990)]. Due to its location in the transit peptide, the precursor form synthesized from either of these start signals would differ only in the length of the transit peptide region and not in the mature protein. Taking into account the high length variability among chloroplast r-protein transit peptides, a shortening of the length need not be deleterious. This view is supported by the fact that the characteristic structural features necessary for transport and chloroplast routing [reviewed in Schmidt and Mishkind (1986)] would still be present in the shorter form.

Initiation of eukaryotic translation has been postulated to occur by a scanning model [recently reviewed in Kozak (1989)]. The fundamental feature of this model is that ribosomes bind to the 5′ terminus of the mRNA, slide toward the 3′ end, and initiate at the first in-context AUG codon. This selection is strongly influenced by the flanking nucleotides of the AUG: a favorable context contains (Kozak, 1986, Lütcke et al., 1987) A or G in position −3 and a G in position +4 (A in AUG is +1). The first AUG codon in all the cDNAs of nuclear-coded chloroplast r-proteins is in this favorable context (Figure 1). Therefore, the two in-frame Met codon arrangement raised the following question: Can the second AUG codon in these mRNAs act as a backup initiation codon for ribosomes that bypassed the first favorable AUG?

In order to test this hypothesis, we constructed a chimeric gene consisting of the 5′ sequence of L12 cDNA and the reporter gene *neo* encoding neomycin phosphotransferase II and introduced frameshifts by site-directed mutagenesis. The expression of the fusion gene was analyzed in vitro, and in vivo in spinach protoplasts. The results are described in this paper.

Materials and Methods

DNA manipulations were done according to standard methods (Maniatis et al., 1982; Berger & Kimmel, 1987). Nucleotide sequence analysis was by the dideoxy method (Sanger et al., 1977), using modified T7 DNA polymerase (Pharmacia). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer (Model 380A) and purified by reversed-phase HPLC on an ODS Hypersil (5 μm) column. The oligonucleotide used for mutagenesis was 5′CATTGTTGTAGTTTGCTGCCATTG3′ (the mutation position is underlined). In vitro transcription was done using CsCl-purified plasmid DNA according to Mead et al. (1986) with T7 RNA polymerase (New England Biolabs) and including ^{m7}GpppG in the reaction mixture. The RNA was translated in a rabbit reticulocyte lysate system (Promega). Neomycin phosphotransferase activity was assayed by the double-gel procedure of Reiss et al. (1984a). The phosphorylated kanamycin was detected by autoradiography; the radioactive spots were cut out and counted in a scintillation counter for quantitation.

Construction of pT7L12-neo. Plasmid pKM109/9, a construct containing the NPT II gene (*neo*) of transposon Tn5 (Reiss et al., 1984b), was digested with *Bam*HI and *Sma*I to yield a fragment of 986 bp. This fragment contains a linker sequence of seven codons and the coding region for neomycin phosphotransferase II but lacks the initiating Met codon. It was ligated into the *Bam*HI and *Hin*cII sites of pT7T318U (Pharmacia), and the construct was named pT7neo. The digestion of L12 cDNA with *Eco*RI and *Hae*III gives a 296 bp fragment containing 78 bp of the 5′ leader, 56 codons of the transit sequence, and 17 codons of the mature L12 protein. It was ligated into the *Eco*RI and *Sma*I sites of pT7neo. Since there is a frameshift between the coding regions in this construct, the DNA was linearized with *Bam*HI; the protruding ends were filled in by Klenow enzyme and religated. The

† This paper is dedicated to the memory of Prof. H. G. Wittmann [for obituary, see *Trends Biochem. Sci. 15*, 332 (1990)].

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r-Protein	Transit peptide N-term. sequence	length	Context of 1st AUG
L12	MAATTTMA...	56	5'...AACAAUG GC...
L13	MATMA...	58	5'...AACAAUG GC...
L35	MAMA...	86	5'...CAUAAUG GC...
PSrp-1	MATLCTSAINMN...	66	5'...AAAGAUG GC...

FIGURE 1: Transit peptides of recently characterized chloroplast ribosomal proteins showing the presence of two Met residues at the beginning of the coding region (see text for references).

addition of four base pairs in this manner makes NPT II in-frame with the two AUG codons in the L12 transit sequence. Positive recombinants were identified by assaying for phosphotransferase activity and confirmed by nucleotide sequencing. The plasmid was named pT7L12-neo.

Site-Directed Mutagenesis of pT7L12-neo. This was done by the gapped duplex method (Kramer et al, 1984) using the uracil selection system (Kunkel et al., 1987). Gapped duplex DNA consisting of single-stranded pT7L12-neo and pT7neo (cleaved with *Eco*RI and *Bam*HI) was annealed with the phosphorylated oligonucleotide KG-1, and the gap was filled by using Klenow enzyme. The annealing and the polymerase reaction were done at room temperature and at 50 °C. Positive clones which contain the mutation were identified by sequencing. At room temperature, the mutation efficiency was >80%, but it included a population of mutants with multiple insertional events. Two of them (L12-neo03 and L12-neo07) were further characterized and used. At 50 °C, the efficiency was 40–50%, and showed only one type of mutant (L12-neo01).

Construction of the p35SL12-neo Series. The coding region of the fusion gene from pT7L12-neo and the mutants were isolated after *Eco*RI and *Hin*dIII digestion and, after filling-in of the protruding ends, were ligated into the *Sma*I site of pUC18A7 (Schaewen, 1989). This vector contains the 35S transcript promoter of cauliflower mosaic virus (Pietrzak et al., 1986) and the polyadenylation signal sequence of the octopine synthetase gene (Gielen et al., 1984). The constructs were named p35SL12-neo, p35SL12-neo01, and p35SL12-neo03. The plasmids pUC18A7 and pKM109/9 were gifts from A. von Schaewen (Institut für Genbiologische Forschung, Berlin).

Expression in Spinach Protoplasts. Protoplasts were made from 2–3-week-old spinach leaves (*Spinacia oleracea*, cv. Matador) by incubating (4–6 h at 25 °C in the dark) with 2% cellulase "Onozuka R-10" and 0.5% macerozyme R-10 (Sigma) in the digestion buffer of Hartung et al. (1980). After filtration through 100-μm nylon mesh, the protoplasts (filtrate) were diluted with 0.5× volume of 0.2 M $CaCl_2$ and centrifuged for 3 min at 100*g* in a swingout rotor. The pellet was suspended in cold W5 solution (Negrutiu et al., 1987), kept at 4 °C for 30 min, centrifuged, and resuspended in the transformation buffer (Negrutiu et al., 1987) to a final concentration of 5×10^6 protoplasts/mL. The suspension was examined under a microscope after fluorescein acetate staining (Widholm, 1972). Plasmid DNA (20–100 μg in 20 μL) was added to 300 μL of the protoplast suspension. It was mixed with an equal volume of 40% PEG [40% poly(ethylene glycol 4000), 0.4 M sorbitol, and 0.1 M $Ca(NO_3)_2$, pH 8.0]. After 30 min at room temperature (transformation), the mixture was diluted with 25 mL of cold W5 solution, and the protoplasts were pelleted. The pellet was suspended in 5 mL of assay buffer (Hartung et al., 1980) and incubated in the dark at room temperature for 20 h for expression. The protoplasts were then harvested and assayed for NPT II activity.

Results

The Fusion Gene. The chimeric gene constructed as described under Materials and Methods encodes a fusion protein that has 73 amino acid residues from the N-terminus of the L12 precursor and 263 amino acid residues from the neomycin phosphotransferase (NPT II) of the transposon Tn5. The N-terminal extension of NPT II would thus contain the complete transit peptide (56 residues) and 17 N-terminus residues of r-protein L12. The NPT II gene in the construct lacks the initiating Met codon, and, therefore, its expression can only be initiated from the first or the second Met codon in the L12 precursor. There are no other Met codons in the construct that can initiate translation.

The different mutant forms of this fusion gene, made by site-directed mutagenesis and used for the expression assay in vitro and in vivo, are shown in Figure 2. The L12-neowt represents the "wild-type": here, both AUG codons in the L12 transit sequence are in-frame with the NPT II coding region. In L12-neo01, only initiation at the second AUG codon can give rise to enzyme activity; the polypeptide initiated at the first AUG codon would, because of the frameshift due to the inserted adenine nucleotide (Figure 2), prematurely terminate

FIGURE 2: Fusion gene L12-neowt and its frame-shifted forms made by site-directed mutagenesis. The inserted base or bases (A in L12-neo01, 17 nucleotides in L12-neo03, and 34 nucleotides in L12-neo07) are indicated by arrows. The N-terminal sequence of the fusion protein initiated from each AUG codon is shown and whether the translation is in-frame or not is indicated. The initiator sequence (cassette) is boxed.

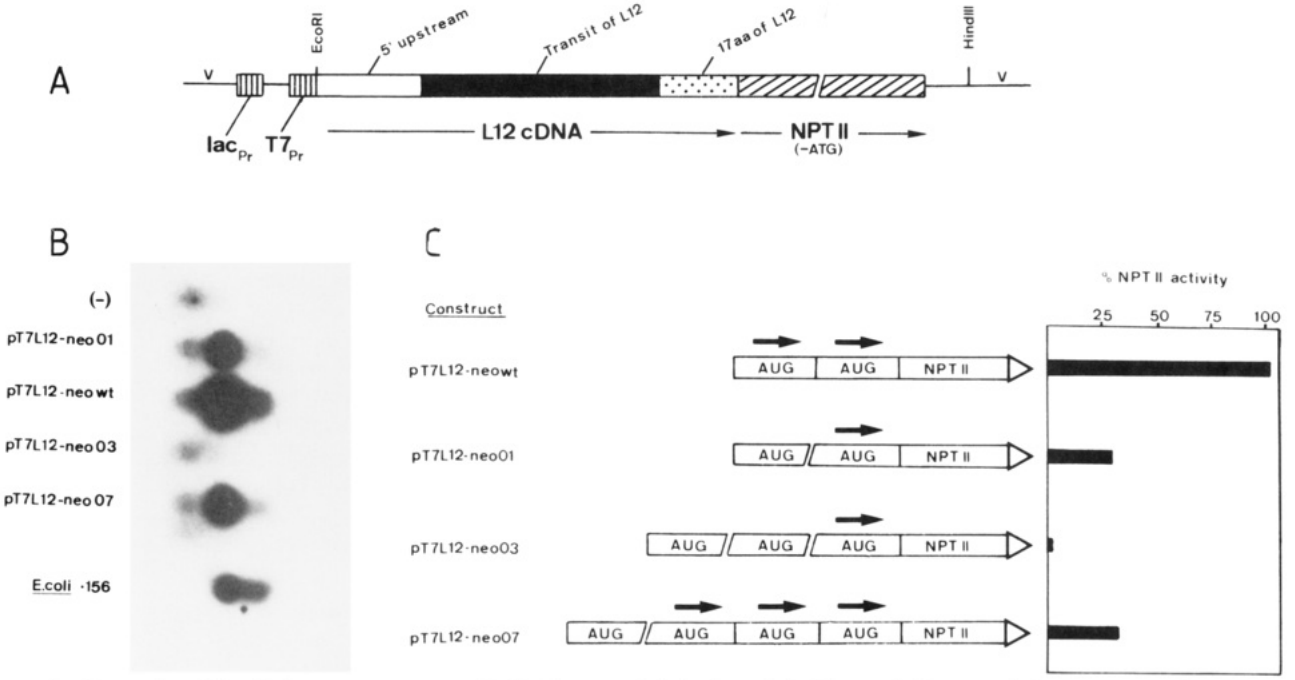

FIGURE 3: Expression of the fusion gene constructs (pT7L12-neo series) in vitro. (A) Schematic diagram of the constructs. Pr, promoter. (B) Assay of the in vitro expressed phosphotransferase activity. (−), control, no RNA added; *E. coli* 156, fusion gene pT7L12-neowt expressed in *E. coli* cells from the lac$_{Pr}$. (C) Quantitative summary of the results.

83 codons downstream. The mutant L12-neo03 has the same frameshift as L12-neo01, but in addition an insert of the primer sequence used for mutagenesis. It therefore contains three AUG codons: two out-of-frame and only one (the third) in-frame and able to initiate the synthesis of the fusion protein. The mutant L12-neo07 corresponds to L12-neo01 in that the first AUG codon is out-of-frame with NPT II, but it contains three copies of the initiation "cassette" AACA*AUG*GC. This construct was used to investigate any enhancement in translation by a reiteration of the initiation context.

In Vitro Analysis of the Fusion Gene Translation. Plasmid DNAs from the pT7L12-neo series were transcribed in vitro, and the mRNAs so synthesized were translated (Materials and Methods). The results from the assay for the NPT II activity of these translation products are summarized in Figure 3. The control (−) which contained all components except any mRNA gave no phosphotransferase activity. The highest enzymic activity was given by pT7L12-neowt. The mutant forms pT7L12-neo01 and pT7L12-neo07 showed, respectively, 26% and 29% enzymic activity, while pT7L12-neo03 showed no detectable activity. A very weak activity in this case could be detected, however, with 3× the amount of the lysate used (data not shown). *Escherichia coli* 156 shows the fusion protein expressed in *E. coli* cells (from the *lac* promoter) by pT7L12-neowt. The presence of three initiation cassettes in pT7L12-neo07 did not result in any significant enhancement of translation: enzymic activities from pT7L12-neo01 and pT7L12-neo07 were approximately the same.

These in vitro results thus showed that a sizable proportion (25–30%) of the ribosomes, which started scanning the mRNA from the 5′ cap, would have bypassed the first AUG codon in the frameshift constructs pT7L12-neo01 and pT7L12-neo07.

Expression of the Fusion Protein in Vivo in Spinach Protoplasts. Although the in vitro system is a convenient tool, it was considered important to confirm this result in vivo. For this purpose, the fusion protein was expressed in spinach protoplasts. This plant was chosen because it provides the homologous system since the L12 cDNA is isolated from a spinach cDNA library (Giese & Subramanian, 1989). Three constructs were analyzed: p35SL12-neowt, p35SL12-neo01, and p35SL12-neo03. Spinach protoplasts were transformed with the construct DNAs, and after expression (Materials and Methods), the protoplasts were lysed and assayed for phosphotransferase activity. The results from these experiments are summarized in Figure 4. The level of expression in the homologous in vivo system was similar to that previously obtained in vitro: translation initiation of ~25% occurred at the second AUG codon in the frameshift mutant p35SL12-neo01 as compared to that (100%) in the unmutated form p35SL12-neowt. The in vitro translation product from the last experiment and a transformation experiment with no added DNA are shown as controls in Figure 4A [pT7L12-neowt and (−)].

The construct with two out-of-frame initiation codons (p35SL12-neo03) gave no detectable activity in the protoplast experiment. The specific requirement for a plant promoter to express the fusion protein in protoplasts was shown by a control experiment where the protoplasts were transformed with pT7L12-neowt DNA. No enzyme activity was detected (shown in Figure 4B).

Discussion

The results described in this paper show that initiation of translation occurs at a significant level from a second AUG codon when there are two in-context AUG codons near the 5′ end of an mRNA. The frequency with which ribosomes initiated at the second AUG codon in our system was approximately the same (25%) when analyzed in vitro or in vivo (Figures 3 and 4). This result suggests that about 25% of the scanning ribosomes fail to stop at the first in-context AUG codon and initiate at the second initiation signal downstream. The only slight translation (~5%) observed at the third AUG codon in the construct pT7L12-neo03 (Figure 3) supports the general nature of this phenomenon, initiation there being presumably from those ribosomes which had also by-passed the second codon.

The construct pT7L12-neo07 with three reiterated downstream AUGs showed no higher level of translation than the

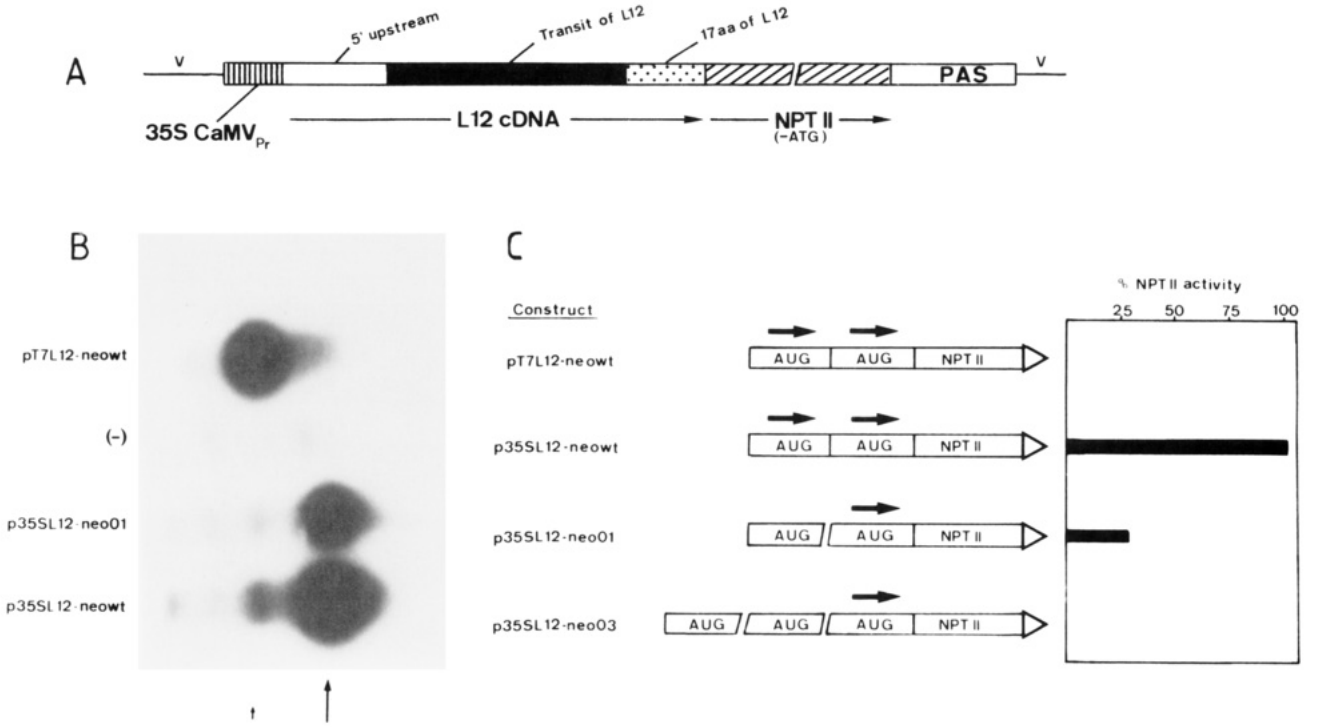

FIGURE 4: Expression of the fusion gene constructs with a plant promoter in spinach protoplasts. (A) Schematic diagram of the constructs. 35SCaMV$_{Pr}$, 35S transcript promoter of cauliflower mosaic virus; PAS, polyadenylation signal sequence. (B) Assay of the in vivo expressed phosphotransferase activity. pT7L12-neowt is a sample from the in vitro experiment (Figure 3). (-), control, no DNA added; the small and the large arrows indicate the assumed precursor and processed forms (see text). (C) Quantitative summary of the results.

construct pT7L12-neo01 with only one downstream AUG (Figure 3). This result and the sharp fall in translation initiated at the first, second, and third AUGs (i.e., 100, 25, and 5%) are in line with the correctness of the basic sliding mechanism (Kozak, 1989) for the initiation of translation.

The electrophoretic mobility of the fusion protein from the protoplast experiment was significantly greater than that of the fusion protein from the in vitro experiment (pT7L12-neowt versus p35L12-neowt in Figure 4A). In a similar experimental situation with the transit sequence of the small subunit of ribulose-1,5-bisphosphate carboxylase fused to the NPT II, van den Broeck et al. (1985) have experimentally shown that the increase in mobility (detected in nondenaturing gels) results from the translocation of the fusion protein into chloroplasts and the consequent transit peptide cleavage. If we assume the fusion protein made in vivo in our experiments has been translocated and processed, the shortening of the transit peptide by six amino acid residues has had no apparent deleterious effects. However, this has to be confirmed.

The first AUG codon in the L12-mRNA corresponds exactly to the plant consensus sequence for functional initiator codons (AACA*AUG*GC; see Lütcke et al., 1987). The by-pass of this initiation signal by 25% of the sliding ribosomes suggests that either initiation at the first in-context AUG codon is perhaps "leaky" or additional features in the mRNA are also involved in the initiation, e.g., the length and nucleotide composition of the leader sequence and the distance/sequence between the two AUG codons. However, in any case, we show that an arrangement of two favorable Met codons in an mRNA enhances the efficiency of translation. It thus appears that the occurrence of two initiation signals in the transit peptide coding region, a common feature among nuclear-coded chloroplast r-protein mRNAs (Figure 1), might be a structural element for achieving a discrete increment in translational efficiency without introducing N-terminal heterogeneity in the mature protein.

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Metabolic Activation of 2-Substituted Derivatives of Myristic Acid To Form Potent Inhibitors of Myristoyl CoA:Protein *N*-Myristoyltransferase†

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ABSTRACT: The importance of myristoylation for the proper biological functioning of many acylated proteins has generated interest in the enzymes of the myristoylation pathway and their interactions with substrates and inhibitors. Previous observations that *S*-(2-oxopentadecyl)-CoA, a nonhydrolyzable methylene-bridged analogue of myristoyl-CoA, was a potent inhibitor of myristoyl-CoA:protein *N*-myristoyltransferase (NMT) [Paige, L. A., Zheng, G.-q., DeFrees, S. A., Cassady, J. M., & Geahlen, R. L. (1989) *J. Med. Chem. 32*, 1665] prompted a closer examination of the effect of substituents at the 2-position on the interactions of myristic acid and myristoyl-CoA analogues with NMT. As an initial approach, three myristic acid derivatives bearing different substituents at the 2-position, 2-fluoromyristic acid, 2-bromomyristic acid, and 2-hydroxymyristic acid, were selected for study. Both 2-bromomyristic acid and 2-hydroxymyristic acid were available commercially; 2-fluoromyristic acid was prepared synthetically. All three compounds were found to be only weak inhibitors of NMT in vitro. Of the three, 2-bromomyristic acid was the most potent (K_i = 100 μM). In cultured cells, however, 2-hydroxymyristic acid was by far the more effective inhibitor of protein myristoylation. Neither 2-hydroxymyristic acid nor 2-bromomyristic acid significantly inhibited protein palmitoylation in cultured cells, indicating that inhibition was not occurring at the level of acyl-CoA synthetase. Activation of the 2-substituted myristic acid derivatives to their corresponding acyl-CoA thioesters by acyl-CoA synthetase resulted in inhibitors of greatly increased potency. The 2-substituted acyl-CoA analogues, 2-hydroxymyristoyl-CoA, 2-bromomyristoyl-CoA, and 2-fluoromyristoyl-CoA, were synthesized and shown to be competitive inhibitors of NMT in vitro (K_i's = 45, 450, and 200 nM, respectively). These data suggested that the enhanced inhibitory potency of 2-hydroxymyristic acid seen in cells was most probably a result of its metabolic activation to the CoA thioester. The presence of substituents at the 2-position also affected the ability of the acyl group to be transferred by NMT to a peptide substrate. Of the three acyl-CoA analogues, only 2-fluoromyristoyl-CoA served as a substrate for NMT.

The covalent attachment of myristate to the amino terminus of proteins was first described for the catalytic subunit of cAMP-dependent protein kinase (Carr et al., 1982) and the B subunit of calcineurin (Aitken et al., 1982). Since these initial discoveries, several additional proteins of both cellular and viral origins have been shown to be covalently modified with myristate [for recent reviews, see Towler et al. (1988a) and Schultz et al. (1988)]. It is the identity of these proteins that has engendered much of the current interest in protein myristoylation. Among others, these proteins include oncogene and protooncogene products such as pp60$^{v\text{-}src}$, pp60$^{c\text{-}src}$, and other members of the *src* family of protein–tyrosine kinases (Marchildon et al., 1984; Towler et al., 1988b), as well as structural proteins encoded by several classes of mammalian retroviruses (Paul et al., 1987; Henderson et al., 1983; Chow et al., 1987; Persing et al., 1987; Streuli & Griffin, 1987). For many of these proteins, myristoylation is essential for proper trafficking and biological function. For instance, nonmyristoylated variants of pp60$^{v\text{-}src}$ retain protein–tyrosine kinase activity but are no longer able to associate with the plasma membrane and are defective in their ability to transform cells (Cross et al., 1984). Likewise, nonmyristoylated variants of the structural proteins of both the human immunodeficiency virus and the Moloney murine leukemia virus do not associate with the plasma membrane and consequently fail to assemble into viral particles (Rein et al., 1986; Göttlinger et al., 1989).

The importance of myristoylation for the proper biological functioning of many acylated proteins has generated interest in the enzymes of the myristoylation pathway and their interactions with substrates and inhibitors. Protein myristoylation is a cotranslational event that occurs following the

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