NAD-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase is targeted to the cytoplasm in insect cell lines

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Abstract Cytosolic NADP-dependent methylenetetrahydrofolate dehydrogenase-cyclohydrolase synthetase and the mitochondrial NAD-dependent methylenetetrahydrofolate dehydrogenase-cyclohydrolase (NMDMC) are differentially expressed during insect development although both enzymes are detectable at all stages. In contrast, cell lines derived from a variety of insect species express high levels of NMDMC but undetectable levels of the NADP-dependent enzyme. Northern analysis indicates the NMDMC message is expressed at levels 50–100 times higher in a *Drosophila* cell line compared to adult flies. RNase protection showed the predominance of shortened transcripts that require initiation at a downstream AUG producing a truncated protein that lacks a mitochondrial targeting sequence. These changes in expression effectively exchange the cytosolic NADP-dependent dehydrogenase for one with NAD specificity.

Key words: Folate; Insect; Mitochondria; Methylenetetrahydrofolate dehydrogenase

1. Introduction

In mammalian cells, the interconversion of one-carbon substituted folates is accomplished by a number of activities, several of which are present as multifunctional enzymes. The trifunctional NADP-dependent methylenetetrahydrofolate dehydrogenase-cyclohydrolase synthetase is localized in the cytoplasm of cells and is responsible for interconverting one-carbon units required for the synthesis of thymidylate, methionine and purines [1]. In addition, an NAD-dependent bifunctional methylenetetrahydrofolate dehydrogenase-cyclohydrolase (NMDMC) is also present but only in mitochondria [2–4]. The cDNA has been cloned from mouse [5] and human [6] libraries and the mouse genomic structure has been determined [7]. In mammals the gene codes for two transcripts of 2.0 and 1.7 kb that arise by differential polyadenylation [5,6]. Recently, the cDNA has been isolated from *Drosophila* which also has two transcripts of 1.35 and 1.25 kb [8]. As is the case in mammals, the *Drosophila* cDNA codes for a protein with a mitochondrial targeting sequence [8]. Although its metabolic role is unproven, its presence at low levels in all adult tissues as well as its complex regulation suggest it could be important in mitochondrial biogenesis by providing the formyl group of formylmethyl-tRNA^{fmet} required for initiation of translation in this organelle [9,10].

Recently, our laboratory initiated an investigation into folate-mediated metabolism of insect cells in culture where the distribution of methylenetetrahydrofolate dehydrogenases was shown to differ from that of mammalian cells [11]. Briefly, we discovered that in the Sf9 cell line derived from *Spodoptera frugiperda* the NADP-dependent dehydrogenase-cyclohydrolase synthetase activities were undetectable by enzyme assay. The absence of this enzyme was further confirmed by the inability of the cells to incorporate radiolabeled formate into purines via the synthetase activity. These were unusual observations since it had previously been shown that the NADP-dependent trifunctional protein is ubiquitously expressed in eucaryotes [1] and functions as a house-keeping gene [12]. However, the cells were still able to generate formyltetrahydrofolate for purine synthesis by expressing an NAD-dependent dehydrogenase-cyclohydrolase. This enzyme, usually present exclusively in mitochondria [4], was localized to the cytoplasm of these insect cells as determined by subcellular fractionation. This NAD-dependent bifunctional enzyme was purified [11] and shown to have very similar properties to its mammalian mitochondrial homologue [3].

In the present study, we have extended our analyses to several other insect cell lines and tissues and propose an explanation for the location of NMDMC in the cytoplasm of insect cell lines.

2. Materials and methods

2.1. Cell lines

Ten continuous insect cell lines, listed in Table 1, were used in these investigations. Unless otherwise specified, all insect tissue culture reagents were purchased from Gibco BRL. Sf9 cells, a clone of the pupal ovarian cell line IPLB-SF-21 AE of *Spodoptera frugiperda* [13], were kindly supplied by Dr. Max Summers (Texas A&M University) and were grown as previously described [14]. *Drosophila melanogaster* S3 cells were a generous gift from Dr. Robert Tanguay (Centre Hospitalier Université Laval) and were grown in Schneider's *Drosophila* medium [15], a modification of her 1964 medium [16]. A moth cell line, American Type Culture Collection (ATCC) CCL 80, developed from the pupal ovaries of *Antheraea eucalypti* [17] and subsequently adapted to a hemolymph-free medium by Yunker et al. [18], was purchased from ATCC. It was grown in Grace's insect tissue culture medium [17] supplemented with 10% fetal bovine serum at 23°C as described by ATCC. The other 7 insect cell lines were developed at the Forest Pest Management Institute (FPMI), formerly Insect Pathology Research Institute (IPRI), from two lepidopteran defoliators of the Canadian

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Abbreviations: NMDMC, NAD-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase; dNMDMC, *Drosophila* NMDMC; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase(s).

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forests. IPRI-CF-1 [19], IPRI-CF-16T, an attached strain of IPRI-CF-16 [19], and IPRI-CF-124T [20] originated from neonate larvae of the spruce budworm, *Choristoneura fumiferana*. FPMI-CF-70 [19], FPMI-CF-27 and FPMI-CF-34 (S.S. Sohi, unpublished results) were developed from pupal ovaries of *C. fumiferana*. The IPRI-MD-66 cell line originated from hemocytes of the forest tent caterpillar, *Malacosoma disstria* [21]. These 7 cell lines were grown at 28°C in Grace's medium [17] supplemented with 0.25% (w/v) tryptose broth (Difco Laboratories, Detroit, MI) and 10% fetal bovine serum.

2.2. Insects

The spruce budworm was reared on a meridic diet [22] after the method of Grisdale [23] at 22°C, 70% R.H. and a photoperiod of 18 h light and 6 h dark. The non-feeding first instar and diapausing 2nd instar larvae were pooled and analyzed. The rest of the 4 larval instars were separately analyzed. Both male and female pupae were pooled whereas the moths were separately analyzed. Tissue samples such as fat body, testes, ovary, integument and hemolymph were from the 6th instar larvae. The fruit flies, *D. melanogaster*, were obtained from Dr. Paul Lasko (Department of Biology, McGill University).

2.3. Enzyme assays

NADP was purchased from Boehringer, Mannheim. NAD, ATP and benzamidine were obtained from Sigma Chemical Co. 2-Mercaptoethanol was obtained from Eastman Chemical Co. All other chemicals were of the highest grade commercially available. Crude cell extracts were prepared as previously described and all enzymatic activities were monitored by spectrophotometric measurements [2]. Protein assays were based on the dye binding method of Bradford [24] using reagents supplied by Bio-Rad Co.

2.4. RNA preparation and Northern analysis

Total RNA was isolated by the acid-phenol/guanidine isothiocyanate method [25]. Formaldehyde-agarose gel electrophoresis was conducted by standard techniques [26] and RNA was transferred to Hybond N (Amersham) using a Bio-Rad vacuum blotter as described by the manufacturer. High specific activity [^{32}P]cDNA probes were generated using random priming [27]. The dNMDMC message was probed using the *D. melanogaster* cDNA (a gift from Dr. Allen Laughon, University of Wisconsin-Madison). GAPDH was probed with the rat cDNA [28]. Hybridization was conducted at 42°C overnight in 5 × SSPE (1 × SSPE: 0.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM EDTA), 35% deionized formamide, 5 × Denhardt's solution, 0.5% SDS and 100 μg/ml boiled salmon sperm DNA with 1×10^6 cpm/ml of the probes. High stringency washes were performed in 0.2 × SSC (1 × SSC: 0.15 M NaCl, 0.015 Na_3 citrate), 0.1% SDS at 60°C for 15 min. Washed membranes were exposed to Kodak X-OMAT AR film.

2.5. Northern analysis with oligonucleotides

Two oligonucleotides were synthesized at the Sheldon Biotechnology Centre, McGill University. Both were 23-mers where *oligo-1* and *oligo-2* had the sequences 5′-CCAGATCGCCGGCCACATCACTC-3′ and 5′-GACTTCTTTTTGTGGCTTTTGCG-3′, respectively. The oligonucleotides were 5′-end labeled using standard techniques [26] with polynucleotide kinase and [γ-^{32}P]ATP (Amersham). RNA from S3 cells was prepared, electrophoresed and transferred to Hybond N as described above and hybridized with the probes at 1.5×10^6 cpm/ml in 6 × SSPE, 5 × Denhardt's solution, 0.1% SDS and 100 μg/ml boiled salmon sperm DNA at 42°C for 8 h. Membranes were washed in 2 × SSC, 0.1% SDS at 42°C for 30 min and exposed to film for 3 days.

2.6. RNase protection assays

All restriction and modifying enzymes used in this analysis were from New England Biolabs, unless otherwise stated. The 5′-end of the dNMDMC message was mapped using an antisense RNA probe transcribed from the dNMDMC cDNA as a template. The cDNA, previously cloned in pBluescript KS$^+$ [8], was digested with *Sac*I which yielded the first 348 base pairs of the cDNA containing the 5′-untranslated region and the first 76 amino acids of the predicted coding region. After purification from an agarose gel by Glassmax (Gibco BRL), the fragment was ligated into pBluescript SK$^+$ (Stratagene). The resulting plasmid was propagated in *E. coli* and isolated by alkali lysis [26]. Clones with the dNMDMC coding region oriented in the direction of the T7 promoter of SK$^+$ were digested with *Xho*I and *Bal*I, removing a fragment containing all the 5′-untranslated region and the first 2 amino acids. After blunting with Klenow in the presence of dNTPs and religation, the resulting plasmid contained the cDNA region coding for amino acids 3–76 of the dNMDMC. Linearization of the template plasmid was carried out with *Dra*II and T3 RNA polymerase was used to produce the antisense probe. The RNA probe was transcribed in the presence of [α-^{32}P]GTP (Amersham), purified and hybridized to total RNA as described by the Promega Applications Guide (Promega Corporation). T7 polymerase was used to produce a sense transcript that was utilized as a positive control for detection of the full-length message. Following nuclease treatment with RNase A and T1 [26], the fragments were electrophoresed on a 6.5% acrylamide gel containing 8 M urea. Dried gels were exposed to Kodak X-Omat AR film overnight.

3. Results

The unusual subcellular localization of NMDMC in Sf9 cells as well as the apparent lack of NADP-dependent dehydrogenase-cyclohydrolase synthetase prompted us to investigate whether the distribution of the enzymes was unique to this cell line. We assayed extracts of cell lines derived from another moth (*A. eucalypti*), *Drosophila* (*D. melanogaster*), spruce budworm (*C. fumiferana*) and forest tent caterpillar (*M. disstria*). As with Sf9, all these insect cell lines (representing both lepidoptera and diptera species) contain the NAD-dependent dehydrogenase activity and none has any detectable NADP-dependent dehydrogenase activity (Table 1).

Several tissues from the spruce budworm were assayed. This

Table 1
Methylenetetrahydrofolate dehydrogenase activities in insect cell lines and tissues

	NAD-dependent (nmoles min^{-1}mg^{-1})	NADP-dependent (nmoles min^{-1}mg^{-1})
Cell lines		
S. frugiperda, ovary		
Sf9	10.4	ND*
D. melanogaster		
S3	7.8	ND
A. eucalypti, ovary		
CCL 80	5.0	ND
C. fumiferana, neonate larval		
IPRI CF1	1.1	ND
IPRI CF16T	4.6	ND
IPRI CF124T	4.0	ND
C. fumiferana, ovary		
FPMI CF27	3.3	ND
FPMI CF34	11.5	ND
FPMI CF70	8.9	ND
M. disstria		
IPRI MD66	1.4	ND
Tissues		
C. fumiferana		
Whole adult	1.1	10.4
Fat body	0.6	29.5
Testes	5.2	4.0
Ovary	1.3	4.6
Hemolymph	ND	ND
Integument	0.7	6.9
D. melanogaster		
Whole adult	1.5	6.3

*ND not detected (<0.2 nmoles min^{-1}mg^{-1}).

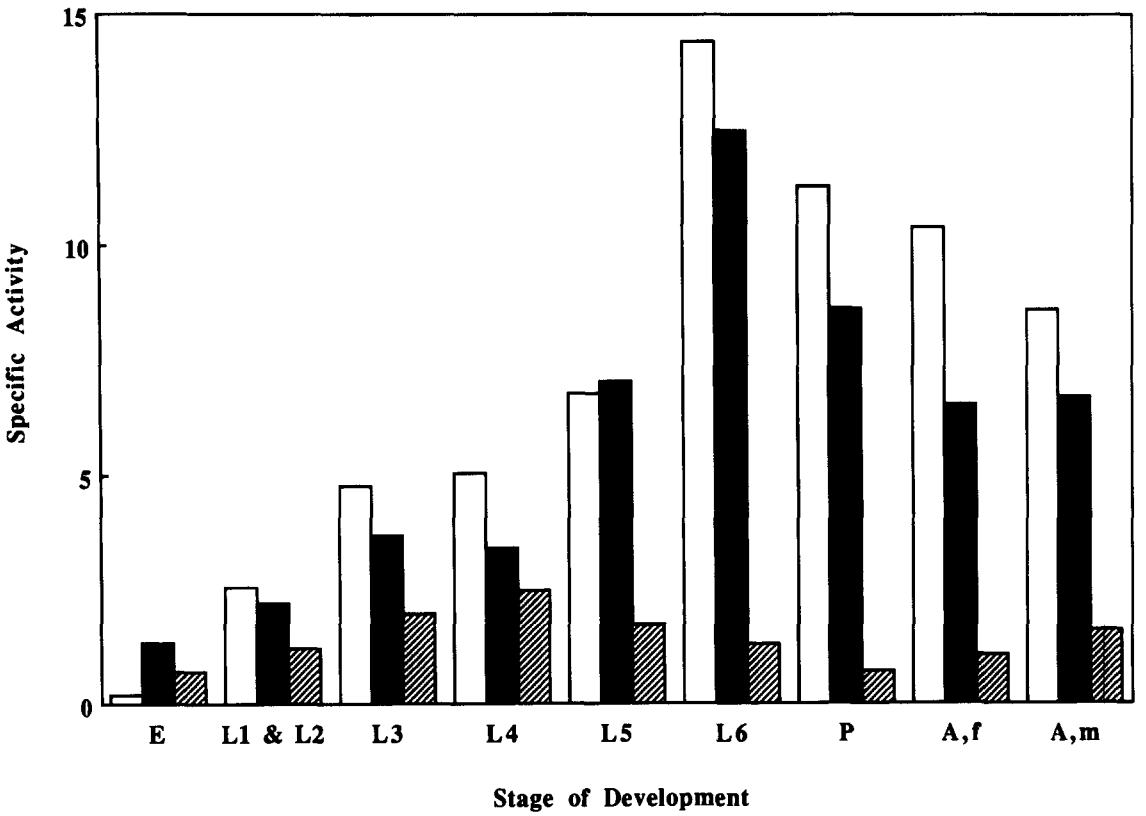

Fig. 1. Developmental expression of the dehydrogenase and synthetase activities in *C. fumiferana*. The specific activity is expressed as nmol·min^{-1}·mg^{-1}. NAD-dependent dehydrogenase is represented by the hatched bars; NADP-dependent dehydrogenase by open bars; formyltetrahydrofolate synthetase by closed bars. The abbreviations are: E, egg; L1–L6, 1st–6th instar larva; P, pupal; A, adult; f, female; m, male.

organism was selected because of its relatively large size and availability. In all tissues except testes and ovaries, levels of NADP-dehydrogenase approximately 10-fold higher than those of the NAD-dependent enzyme were observed (Table 1). No activity was detected in hemolymph. In all cases the formyltetrahydrofolate synthetase activity was assayed and, as expected, it could be detected only in tissues containing the NADP-dependent dehydrogenase activity (data not shown).

We wanted to determine if these insect methylenetetrahydrofolate dehydrogenases were developmentally regulated and assayed samples of tissues from different developmental stages of spruce budworm. As shown in Fig. 1, the NAD-dependent dehydrogenase acquires maximal activity at the 4th instar larva whereas the NADP-dependent dehydrogenase is highest in the 6th instar larva. In the pupal and adult stages, the NADP-dependent enzyme activity remains relatively high whereas the NAD-dependent activity decreases. Again, at all stages except egg, the synthetase activity correlated well with the NADP-dependent dehydrogenase activity.

In an attempt to elucidate how the NAD-dependent dehydrogenase-cyclohydrolase in insect cell lines is located in the cytoplasm instead of the mitochondria, we examined the mRNA encoding this protein. We pursued our analysis in *D. melanogaster* since the cell line, S3, derived from this insect has the same distribution of methylenetetrahydrofolate dehydrogenases as the Sf9 cell (Table 1). Moreover, the cDNA for *Drosophila* NMDMC has recently been obtained from an embryonic cDNA library and contains the full length amino acid sequence including the mitochondrial leader sequence [8].

Total RNA from the *Drosophila* S3 cell line and adult flies was prepared and used in a Northern analysis. As shown in Fig. 2, the expected 1.3 kb dNMDMC message is present at much higher levels in the cell line compared to the adult tissue. The amount of RNA was normalized using rat GAPDH cDNA [28] as a probe (Fig. 2) and shown to be equal in each lane. Although the dNMDMC message in the S3 lane can be seen easily after an overnight exposure (data not shown), detection of the message in the adult tissue lane required 7 days of exposure.

To determine if the sequence coding for the mitochondrial targeting signal was present in dNMDMC message in S3 cells, two antisense oligonucleotides were synthesized to probe total S3 RNA in a Northern analysis. *Oligo-1* is complementary to the 5′-end of the mitochondrial targeting signal whereas *oligo-2* is complementary to the 3′-end (see Fig. 3C). As shown in Fig. 3A, the dNMDMC message in the S3 cell line could only be detected with *oligo-2*. The RNA was again normalized with the rat GAPDH probe and shows that equal amounts of RNA were present in each lane. Both oligonucleotides hydridized to the dNMDMC cDNA when tested in a Southern analysis (data not shown).

The previous observation suggested that the 5′-end of the mRNA is located somewhere in the sequence delineated by *oligo-1* and *oligo-2*. To test this hypothesis, an RNase protection assay was conducted using S3 RNA to see if any messages

started within this region. An RNA probe was synthesized using as template a fragment of the dNMDMC cDNA coding for the mitochondrial targeting sequence as well as the first 23 amino acids of the mature protein. When the labeled RNA probe was hybridized to 10 μg of S3 RNA from the cell line, two major protected bands were seen with sizes of 122 and 118 bases (see Fig. 3B). The location of these transcriptional start points would produce messages beginning approximately 105 bases into the coding region of the mitochondrial targeting sequence (see Fig. 3C). The labeled probe was also hybridized to 60 μg of yeast tRNA as a negative control and no bands were detected.

4. Discussion

Mammalian cells express the NMDMC protein in mitochondria where it is presumed to provide the precursor for formylmethionyl-tRNA^{fmet} required for initiation of protein synthesis [10]. Our previous results indicated that this enzyme is expressed in the cytoplasm of Sf9 cells derived from the fall army-worm [11]. Subcellular fractionation of the *A. eucalypti* cell line (data not shown) indicated that the NAD-dependent dehydrogenase was localized to the cytoplasm as in the Sf9 cell indicating that this localization is the same in all cell lines we have analyzed. Coupled with the undetectable levels of the NADP-dependent dehydrogenase-cyclohydrolase synthetase, we suggested that the function of NMDMC in the cytoplasm of these cells is to provide one-carbon units required for purine biosynthesis. In the present paper, we report that the situation originally observed in Sf9 cells seems to be a general characteristic shared by other insect-derived cell lines. In all cases, the NAD-dependent bifunctional enzyme is highly expressed in the cytoplasm and the NADP-dependent trifunctional enzyme is undetectable.

Tissues of spruce budworm (*C. fumiferana*) contained levels of NADP-dependent enzyme approximately 10-fold higher than the NAD-dependent dehydrogenase. Fat body which has a function similar to that of liver in mammals, has very high levels of the NADP-dependent trifunctional protein. This is consistent with the results seen in mammalian liver where this enzyme is also expressed at high levels [2,29]. Elevated levels of NMDMC mRNA were reported in murine testes [9]. Interestingly, spruce budworm testes expressed the highest levels of enzyme activity among the tissues we have analyzed. Although these results are consistent with enzyme levels in mammalian tissues where NMDMC is either very low or undetected [2], all insect tissues had detectable levels of the enzyme. The reason for which higher levels of NMDMC are required in insect tissues is unknown.

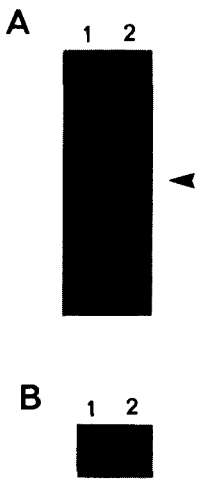

Fig. 2. NMDMC levels in S3 cells and adult *D. melanogaster*. (A) 10 μg/lane of total RNA from the S3 cell line (lane 1) and adult fruit flies (lane 2) was probed with the entire dNMDMC cDNA. The arrowhead indicates the position of the 1.3 kb message. (B) hybridization of the same blot with the rat GAPDH probe to control for RNA quantity.

The spruce budworm was also used to study the levels of each enzyme during development. NMDMC is expressed at all stages with the activity reaching its highest levels at the 4th instar larva. Results obtained with the *Drosophila* NMDMC message indicated the expression followed a pattern during development of the fruit fly [8] that is similar to this pattern of expression in spruce budworm. We also observed slightly higher levels of NMDMC in males compared to females which was again consistent with mRNA levels in *D. melanogaster* [8]. As is the case in mammals where NMDMC is tightly regulated [9], our results suggest that the insect NMDMC is also regulated as its expression varies among different tissues and during development. The NADP-dependent dehydrogenase-cyclohydrolase synthetase also seems to be developmentally regulated during insect development. The levels of trifunctional enzyme are highest in the 6th instar larva which is later in development than the peak level of NMDMC, suggesting that these methylenetetrahydrofolate dehydrogenases have different metabolic roles in insect development.

The availability of the S3 cell line derived from *D. melanogaster* and the dNMDMC cDNA allowed us to address the unusual localization of NMDMC at the level of the mRNA. A Northern blot conducted on mRNA isolated from S3 cells and adult flies indicates that dNMDMC is expressed at much higher levels in the cell line. The low level of expression as well as the presence of a mitochondrial targeting sequence in the dNMDMC cDNA [8] are consistent with the protein being expressed exclusively in mitochondria in adult flies. In addition, the NAD-dependent dehydrogenase activity is expressed at very low levels (1.52 nmol·min^{-1}·mg^{-1}) in cellular extracts of adult *Drosophila*, which is in agreement with the low levels of NMDMC mRNA we observed.

The results from the Northern analysis suggest that the cytoplasmic localization of NMDMC in the cell line could be due to transcriptional regulation and production of an altered mRNA. To test this possibility, we proceeded to see if the dNMDMC messages contained sequences coding for a mitochondrial leader sequence as predicted by the cDNA. Northern analysis and RNase protection indicated that the S3 cell produces 5′-truncated mRNAs encoding the NMDMC. We were unable to detect a longer transcript that could code for a protein with a signal sequence presumably because of its very low abundance in S3 cells. A sense transcript was hybridized to the labeled antisense probe as a positive control and indicated that the full length message would have been easily detectable under our experimental conditions (data not shown). A methionine residue located at position 55 of the predicted dNMDMC amino acid sequence [8] is the first AUG codon in the shorter transcripts. We propose translation is initiated at this codon to produce a protein that does not contain a mitochondrial targeting sequence and therefore remains in the cytoplasm. In addi-

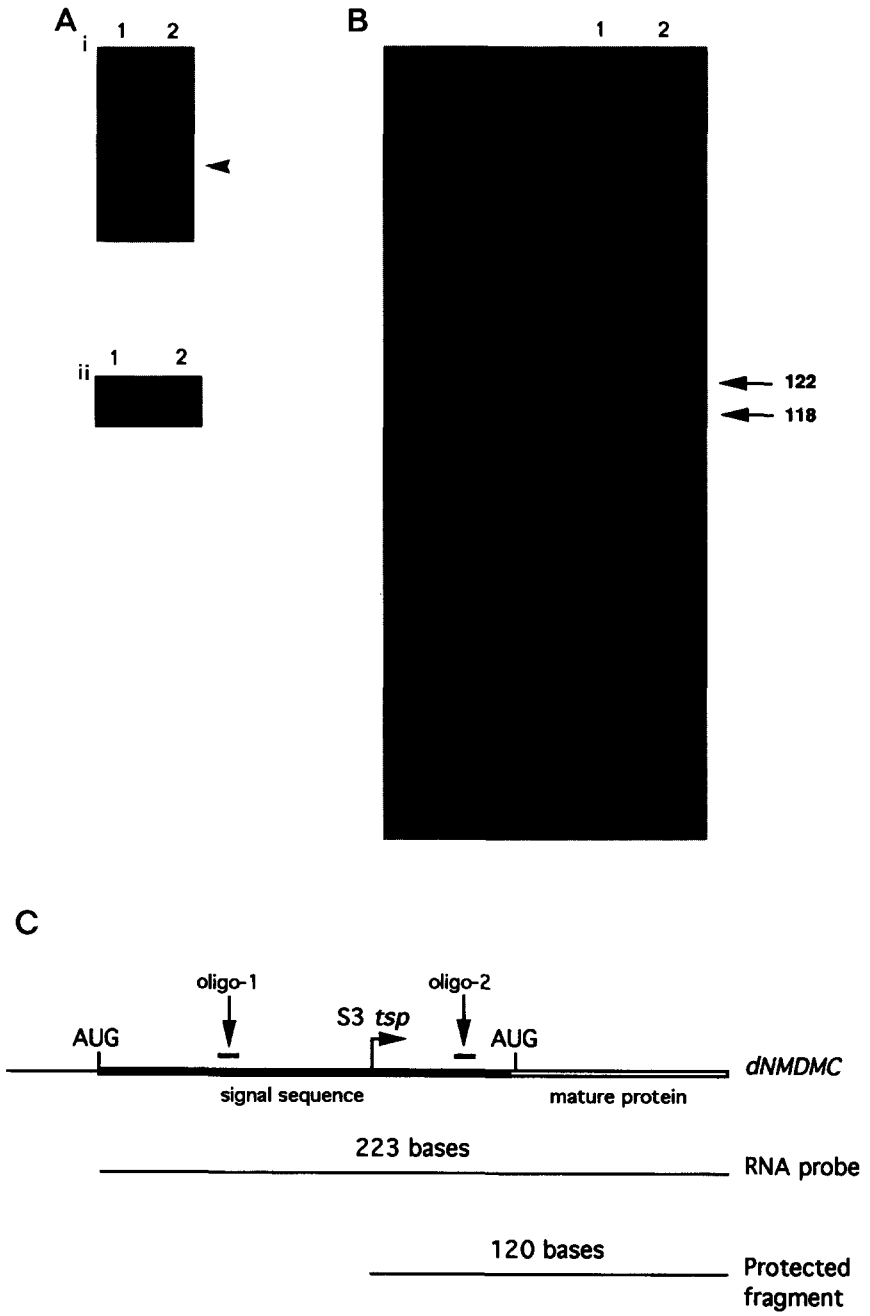

Fig. 3. Mapping the 5′-end of the dNMDMC message in S3 cells. (A_i), Northern analysis of 5′-end of the dNMDMC message from S3 cells. Each lane contains 10 μg of S3 total RNA. Lane 1 and lane 2 indicate hybridization with *oligo-1* and *oligo-2*, respectively. The NMDMC message is indicated by an arrowhead. The blot was exposed for 3 days. The lower panel (A_{ii}) represents the same blot reprobed with GAPDH. (B) RNase protection assay on S3 cell total RNA. Lane 1 shows the transcripts from 10 μg S3 RNA with the arrows indicating the two major transcripts of 118 and 122 base pairs. Lane 2 is the negative control using 60 μg tRNA. The sequence ladder of pBluescript-SK$^+$ vector DNA is used as size markers. (C) schematic representation of the 5′-end of dNMDMC. The S3 trancriptional start point (tsp) is indicated by a right angle arrow.

tion, this AUG has flanking sequences that match both Kozak [30] and *Drosophila* [31] consensus sequences for initiator codons and initiation at this position would produce a protein virtually identical to that located in the mitochondria. Probably the strongest evidence to support initiation at this methionine resides in the fact that 6 of the 7 residues following this methionine are identical to those at the N-terminus of the *Escherichia coli* [32] and *Photobacterium phosphoreum* (P.D. Pawelek and R.E. MacKenzie, unpublished results) dehydrogenase-cyclohydrolases (see Fig. 4). Although the immediate upstream region of these shorter transcripts does not seem to contain typical sequence motifs found in eucaryotic promoters [33], there is a sequence corresponding to a cap-site where the transcriptional start point is located [34].

The dNMDMC gene structure as determined by Price and Laughon contains 3 exons where exon 1 includes the 5′-untranslated region and the entire coding region for the mitochondrial targeting sequence [8]. Our results did not rule out the

Fig. 4. Alignment of N-terminal sequences of methylenetetrahydrofolate dehydrogenase-cyclohydrolases. The amino acid position is indicated to the left of the sequences. The identical residues are in bold.

possible presence of an upstream alternatively spliced exon that contains a 3′-sequence identical to that of exon 1. To address this possibility, *Drosophila* genomic DNA was digested with a restriction enzyme (*Eco*RI) with a site located immediately upstream of the sequence homologous to *oligo-1* (see Fig. 3C). This digested DNA was used in a Southern analysis and probed with *oligos-1* and *-2*. The results showed that both oligonucleotides hybridized to single genomic fragment of 6 kb, indicating that there is only one exon encoding the 5′-end of the dNMDMC gene (data not shown). Furthermore, these results also indicated that there is only one copy of the NMDMC gene in *Drosophila*.

The results in this report provide an explanation for how this protein is localized to the cytoplasm in cell lines derived from insect tissues. However, the regulation involved to achieve this and how the cells turn off the NADP-dependent trifunctional enzyme still remain questions. It is also not clear as to whether these two events are coordinated for some physiological purpose. The result of the changes is to alter the cofactor (NADP to NAD) used by the dehydrogenase in the cytoplasm and would be predicted to increase the ratio of formyl- to methylenetetrahydrofolate in that compartment. The system appears more complicated than simply using one gene to provide the same protein for two cellular compartments, although a similar mechanism is employed in such cases [35,36]. It is possible that these cells lack insect-specific factors that cannot be provided by the fetal bovine serum when in culture. The insect steroid-hormone, ecdysone, has been shown to enhance the expression of several genes when added to insect cell lines in culture [37]. It had been reported that ubiquitin-protein conjugates are enriched in lysosomes of Sf9 cells when they were grown in the presence of ecdysone, indicating that these cells also respond to this hormone [38]. The distribution of NAD- and NADP-dependent methylenetetrahydrofolate dehydrogenases remained unaffected when we grew the Sf9 cells in the presence of ecdysone, indicating that it did not alter the expression of these genes (data not shown). Nevertheless, our results demonstrate that the expression of these methylenetetrahydrofolate dehydrogenase genes can be drastically altered and raises new questions as to the nature of their regulation.

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