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Transcriptional regulation of murine NADP$^+$-dependent methylenetetrahydrofolate dehydrogenase-cyclohydrolase-synthetase

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The cytosolic NADP$^+$-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase is ubiquitously expressed in all mouse tissues and cell lines examined. Northern analyses of the RNA indicated that there is an extensive variation in the levels of mRNA in different tissues. However, the gene is refractory to induction by serum, phorbol esters or growth factors in cultured fibroblasts. The mRNA of the NADP$^+$-dependent trifunctional enzyme is stabilized post-transcriptionally by insulin-like growth factor-1.

Folate; NADP$^+$-dependent methylenetetrahydrofolate dehydrogenase; Trifunctional enzyme; Multifunctional enzyme; IGF-1; Post-transcriptional regulation

1. INTRODUCTION

The cytosolic trifunctional folate enzyme, NADP$^+$-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase catalyses three sequential metabolic reactions, interconverting 10-formyltetrahydrofolate, which is used in purine biosynthesis, and methylenetetrahydrofolate, which is used either in synthesis of thymidylate or, after reduction, in the formation of methionine [1]. This enzyme has been assayed in various mammalian tissues and cell lines and found to be ubiquitously expressed [2,3]. The cDNA of the human trifunctional enzyme has been cloned, sequenced [4] and the domains comprising the dehydrogenase-cyclohydrolase and the synthetase activities have been expressed in *Escherichia coli* and characterized [5]. The gene encoding the rat trifunctional enzyme has been cloned and the mRNA and the protein levels have been shown to vary by as much as 10-fold among different tissues; highest levels are found in kidney and liver [6]. Here we report that the murine trifunctional enzyme, although expressed at different levels in various mouse tissues, is not induced by serum or growth factors in cultured mouse fibroblasts; however, the mRNA is stabilized post-transcriptionally by IGF-1.

2. MATERIALS AND METHODS

2.1. *Materials*

Cell culture media, fetal bovine serum and antibiotics were purchased from Gibco Life Technologies. Platelet-derived growth factor (PDGF) and insulin-like growth factor-1 (IGF-1) were from Collaborative Research. Phorbol myristyl 13-acetate (PMA), bovine serum albumin (BSA), actinomycin-D and epidermal growth factor (EGF) were obtained from Sigma. All other chemicals used were of reagent grade. [α-^{32}P]dCTP and HyBond Nylon membranes were purchased from Amersham.

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2.2. *Cell culture*

NIH 3T3 and Balb/C 3T3 (clone A31) cells were obtained from American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. The cells were grown to confluence in 100 mm dishes and then deprived of growth factors by maintaining them in 0.5% fetal bovine serum for 24–48 h. The cultures were then induced by the addition of serum (20%) or growth factors (PDGF 5 ng/ml; EGF 100 ng/ml; PMA 100 ng/ml). BSA (0.5 mg/ml) was added as a stabilizing agent to the medium that contained growth factors.

2.3. *RNA isolation and analysis*

Total RNA was isolated from fresh tissues of male Swiss albino mice (30 g), using the acid-phenol/guanidium isothiocyanate method [7]. PolyA$^+$ RNA was isolated following the method of Aviv and Leder [8]. Radiolabelled probes were prepared by the random primer labelling method [9]. Formaldehyde-agarose gels, transfer of RNA to Hybond nylon membranes by vacuum blotting, prehybridization and hybridization were done as described [10]. The stringent wash of the membranes was done in 0.1× SSPE (0.018 M sodium chloride, 1 mM sodium phosphate, pH 7.4, 0.1 mM EDTA) and 0.1% sodium dodecyl sulfate at 55°C for 30 min. The autoradiograms were exposed to Kodak XO-mat XAR film overnight.

2.4. *Quantitation of gels and autoradiographs*

A Milligen/Bioresearch image analyzer was used to scan and integrate the area corresponding to bands on radioautographs and ethidium bromide-stained DNA on photographs of gels.

3. RESULTS

3.1. *Tissue distribution of the mRNA for the trifunctional enzyme*

In order to investigate the abundance of the trifunctional enzyme in different tissues, 2 μg of polyA$^+$ RNA

was denatured and electrophoresed on formaldehyde-agarose gels. The Northern blots were probed with a 2 kb *Eco*RI cDNA fragment of the human trifunctional enzyme [4]. The Northern blots revealed a single species of mRNA, 3.0 kb in length, similar in size to those encoding the human and the rat trifunctional enzymes [4,6]. The homology between the murine and the human trifunctional enzymes is expected to be high because strong signals were obtained even after the membranes were washed at a high stringency.

It is evident from Fig. 1A that the mRNA levels of the murine trifunctional enzyme in various tissues are different. Both kidney and liver contain high levels of the mRNA as compared to other tissues. The consistency observed in the levels of the actin mRNA indicate that equivalent amounts of RNA were loaded in each lane.

3.2. *Effects of mitogens on the levels of mRNA*

We investigated the effect of mitogens on the transcriptional status of the gene. Confluent monolayers of NIH 3T3 cells were maintained in 0.5% serum for 24–48 h, during which time they were stable but did not divide. Cultures were then supplemented with serum or growth factors as indicated in section 2 to determine if the return to growth and division elevates the steady-state level of the gene encoding the trifunctional enzyme. Total RNA was isolated at various times and was subjected to Northern analysis. Twenty micrograms of the total RNA was loaded into each lane and the consistency among the lanes was verified by the intensities of the 28 S and 18 S RNA bands in the ethidium bromide-stained gel. In addition, the blots were probed with a cDNA fragment encoding glyceraldehyde 3-phosphate dehydrogenase. As seen from Fig. 1B, the level of the mRNA of the $NADP^+$-dependent trifunctional enzyme did not change upon incubation in low levels of serum, nor did it increase after the addition of serum. Similar results were obtained when serum-deprived cells were exposed to growth factors or phorbol esters (data not shown). We repeated these same experiments with another well-characterized mouse fibroblast cell line, Balb/C 3T3 (clone A31) and obtained similar results.

3.3. *Post-transcriptional regulation of the enzyme in mouse fibroblasts*

In contrast with the effects seen in 0.5% serum, when the monolayers were maintained in DMEM containing 0.5 mg/ml of BSA for 3–4 h, conditions under which they cannot be maintained long term, there was a significant reduction in the intensity of the signal of the trifunctional enzyme. We determined whether the mRNA stability could be regulated by post-transcriptional mechanisms while transcription was inhibited by actinomycin D. Confluent Balb/C 3T3 monolayers, a cell system that has been characterized with respect to the post-transcriptional regulation by IGF-1 [13], were

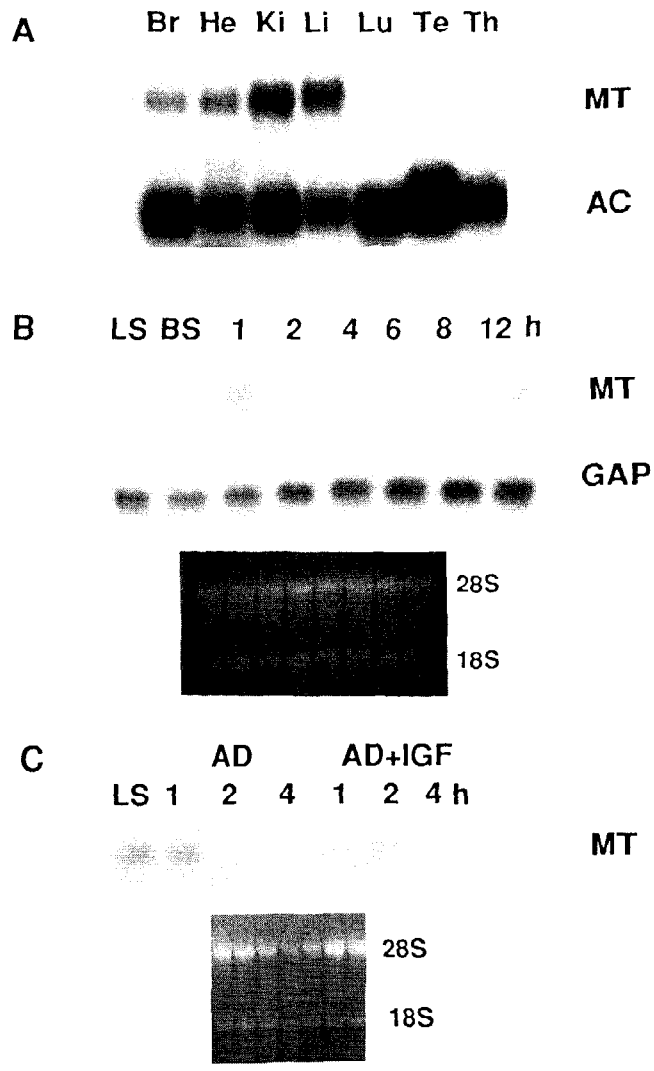

Fig. 1. (A) Tissue distribution of $NADP^+$-dependent trifunctional enzyme (MT) and actin (Ac) mRNAs: Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Te, testis; Th, thymus. (B) Transcription of MT in quiescent NIH 3T3 fibroblasts upon serum stimulation: LS, low serum (0.5%); BS, BSA at 0.5 mg/ml. Total RNA was extracted 4 h after addition of BSA. The ethidium bromide-stained agarose gel showing the 28 S and 18 SrRNA bands is shown in the bottom panel. (C) IGF-1 mediated stabilization of MT mRNA: AD, actinomycin-D; IGF-1, insulin-like growth factor-1. The bottom panel shows the ethidium bromide-stained agarose gel containing 28 S and 18 S rRNA bands.

maintained in 1% serum for 48 h before they were treated with DMEM containing BSA (0.5 mg/ml) and actinomycin-D (20 μg/ml), or BSA, actinomycin-D and IGF-1 (100 ng/ml). Total RNA extracted at various times was analysed in a Northern blot (Fig. 1C). Addition of actinomycin-D prevented de novo transcription so that the decrease in the intensity of the 3.0 kb band represented only the degradation of the mRNA of the trifunctional enzyme. Quantitative image analysis allowed normalization of MT mRNA relative to the ribosomal RNA bands. After 2 h, when normalized relative to 28 S RNA, there was a 4-fold decrease in MT mRNA

and an 8-fold decrease when normalized relative to 18 S RNA. After 4 h, the MT mRNA was undetectable. When this experiment was carried out in the presence of IGF-1 for 2 h, the decrease in MT mRNA was reduced to to only 2-fold when normalized to 28 S RNA and to 1.6-fold when normalized to 18 SRNA. IGF-1 decreased the loss of message due to mRNA turnover as evidenced by the persistence of significant levels of the mRNA of the trifunctional enzyme in the presence of actinomycin-D.

4. DISCUSSION

Our Northern analyses are in agreement with the reported tissue distribution of the protein and the mRNA of the rat trifunctional enzyme [3,6]. High levels of the trifunctional enzyme have also been reported in bone marrow and adrenal cortex [11]. On the basis of the strong correlation between the levels of the mRNA and the protein, Thigpen et al. [6] proposed that the regulation of the trifunctional enzyme is predominantly at the pretranslational level. The tissue distribution was similar whether the analyses used total RNA [6] or polyA$^+$ RNA as in this study. Hence it appears that differential polyadenylation of tissue mRNAs, as proposed in the post-transcriptional regulation of glyceraldehyde 3-phosphate dehydrogenase [12], may not be a significant factor in this case.

At the transcriptional level, we determined that the gene for the trifunctional enzyme is not inducible in mouse fibroblasts by mitogenic agents. This is supported by the fact that the activity of the enzyme does not change upon tumorigenic transformation of rat liver-derived epithelial cells by oncogenes or aflatoxin [11]. Hence the variation in the levels of the trifunctional enzyme in tissues must be influenced by tissue-specific factors. A notable example of a house-keeping enzyme that shows tissue-specific variation but not induction by mitogenic events, is glyceraldehyde 3-phosphate dehydrogenase [12].

One mode of regulation that does affect the mRNA level of the enzyme is the enhanced stability of the mRNA in low serum or in the presence of IGF-1. In a preliminary analysis Zumstein and Stiles [13] obtained several cDNA clones from Balb/C 3T3 cells, which represented constitutively expressed genes, the transcripts of which are protected by IGF-1 from nucleolytic degradation. Notable among them was pI-15, whose stability was positively correlated with the survival of the cells at high density. Other relevant examples of mRNAs whose stability is influenced by growth factors, by the growth status of the cell or by differentiation are c-myc, c-myb and p53 [14,15]. Similarly, thymidine kinase [16], tubulin [17], and glyceraldehyde 3-phosphate dehydrogenase [12] are regulated by post-transcriptional mechanisms. In the case of the NADP$^+$-dependent trifunctional folate enzyme, modulation of the mRNA stability by IGF-1 or related proteins appears to be an important regulatory mechanism.

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