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Alterations of Gene Expression in Endoderm Differentiation of F9 Teratocarcinoma Cells

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ABSTRACT During the endoderm differentiation of F9 mouse embryonal carcinoma cells, as induced by sodium butyrate (NaBu) or retinoic acid (RA), gene expressions of alkaline phosphatase (ALPase), pyruvate kinase (PKase) and 5′ ribonucleotide phosphohydrolase (5′-Nase) were examined. The specific activity of ALPase was found to increase by 3.5-fold after 48 hr treatment with NaBu. In contrast, specific activity of PKase were decreased by 63%. Northern blot analysis revealed that the elevation of ALPase activity resulted from an increase in the level of liver/bone/kidney (L/B/K)-type ALPase mRNA and that the decrease of PKase activity was dependent on a reduction in the level of M_2-PKase mRNA. Interestingly, when NaBu was removed from the culture medium, the levels of these mRNAs reverted to their original levels after 16 h. During these processes, the specific activity of 5′-Nase and the level of its mRNA remained unchanged. In contrast, when F9 cells were treated with RA, only the level of L/B/K-type ALPase mRNA increased. Lastly, we examined the issue of whether an increase in the level of ALPase mRNA is dependent on the transcriptional activation of the mouse L/B/K-type ALPase gene. Transient transfection assays using luciferase reporter constructs showed that the promoter activity increased as the result of treatment with RA but not with NaBu. *Mol. Reprod. Dev. 60: 165–171, 2001.* © 2001 Wiley-Liss, Inc.

Key Words: alkaline phosphatase; 5′-nucleotidase; pyruvate kinase; gene expression; cell differentiation; transcriptional control

INTRODUCTION

F9 cells, a mouse embryonal carcinoma cell line, represent a useful model system for the study of the mechanism(s) of endoderm differentiation in mouse early embryogenesis (Strickland and Mahdavi, 1978; Strickland et al., 1980; Kosaka et al., 1991, 1992). When F9 cells, on treatment with retinoic acid (RA), differentiate into primitive endoderm in monolayer cultures or into proximal (visceral) endoderm cells in aggregation culture (Strickland and Mahdavi, 1978). In addition, F9 cells are also able to differentiate into distal (parietal) endoderm cells, on treatment with RA and dibutyryl cyclic AMP in monolayer culture (Strickland et al., 1980). It has also been reported that sodium butyrate (NaBu), a weak inhibitor of histone deacetylase, also induces endoderm differentiation of F9 cells (Kosaka et al., 1991). While these differentiation-induced cells express a number of endoderm cell-specific marker molecules, including α-fetoprotein, plasminogen activator, laminin B1 chain, and cytokeratin endo A mRNAs, the level of c-myc mRNA as an undifferentiated cell marker molecule decreases, after a period of time (Strickland and Mahdavi, 1978; Strickland et al., 1980; Kosaka et al., 1991, 1992). When NaBu but not RA is withdrawn from a differentiation-induced medium, the disappearance of the endoderm-specific molecules and reversion of the level of c-myc mRNA occurs (Kosaka et al., 1991). Therefore, it would appear that RA-treated F9 cells exhibit irreversible differentiation via determination but that differentiation in the case of NaBu-treated F9 cells is reversible.

Alkaline phosphatase (ALPase, orthophosphomonoester phosphohydrolase, EC 3.1.3.1) and 5′-ribonucleotide phosphohydrolase (5′-Nase, EC 3.1.3.5), both of which are localized in the cell membrane, catalyze the hydrolysis of phosphomonoester bonds as well as of the sugar-phosphate backbone of nucleotide, respectively. Three ALPase isoenzymes have been identified in rodents, liver/bone/kidney (L/B/K)-, embryonic-, and intestine-types (Nair et al., 1987a,b; Hahnel et al., 1990). Pyruvate kinase (PKase, ATP: pyruvate O^2-phosphotransferase, EC 2.7.1.40), a rate-controlling cytosolic glycolytic enzyme, catalyses a production of pyruvate and ATP from phosphoenolpyruvate and ADP (Yamada and Noguchi, 1999a,b). Whereas four isoenzymes of PKase have been identified in mammals, M_2-

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PKase is the only isoenzyme present in embryo and early fetal tissues (Yamada and Noguchi, 1999b). In a previous study, we showed that the specific activity of ALPase increased considerably during the endoderm differentiation of RA-treated F9 cells, but that 5′-Nase activity increased only temporarily (Yamada et al., 1992). We and others also reported that the L/B/K-type ALPase mRNA is originally expressed in F9 cells, that its expression is induced in RA-treated F9 cells but an increase of the level of its mRNA preceded the observed elevation of ALPase activity (Gianni et al., 1991; Yamada et al., 1992).

The focus of the present study was to examine alterations of the specific activities of ALPase, PKase, and 5′-Nase and the expression of their mRNAs during the endoderm differentiation of F9 cells, as induced by treatment with NaBu. We further examined the issue of whether an alteration of the level of L/B/K-type ALPase mRNA during the endoderm differentiation of F9 cells is dependent on the regulation of promoter activity of the mouse L/B/K-type ALPase gene.

MATERIALS AND METHODS

Materials

A Bio-Rad protein assay kit was obtained from Bio-Rad. The TRIZOL reagent, Superscript II, and LIPOFECTAMINE PLUS were purchased from GIBCO/BRL. The Ex Taq and BcaBest DNA labeling kit were purchased from Takara. The rTaq DNA polymerase was obtained from Toyobo. The QIAEXII gel extraction kit and QIAGEN plasmid kit were purchased from QIAGEN. The pGEM T-Easy, pGL3-Basic, pGL3-Control, and pRL-CMV vectors and dual luciferase reporter assay system were purchased from Promega (Madison, WI). The Biodyne A membrane was obtained from PALL. The Thermo Sequenase II dye termination cycle sequencing kit were purchased from Amersham Pharmacia Biotech. α-^{32}P dCTP (111 TBq/mmol) was obtained from Du Pont/New England Nuclear.

Cells and Cell Culture

F9 cells were obtained from the Department of Cells and Viruses of the Institute for Virus Research, Kyoto University. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics at 37°C in a 5% CO_2 incubator. For the induction of endoderm differentiation, F9 cells, in a monolayer culture, were treated with 5 mM NaBu or 2×10^{-7} M all-*trans* RA.

Assays of Enzyme Activities

F9 cells were plated at 2×10^6 cells/100 mm-diameter culture dish. After 24 hr, the medium was changed to either a normal medium or a differentiation medium. Cells were harvested at various times and the enzyme activities and protein concentration were determined. Assays of enzyme activities of ALPase and 5′-Nase have been described previously (Yamada et al., 1992). The method of Imamura and Tanaka (1982) was used for the assay of PKase activity. Protein concentration was determined using the Bio-Rad protein assay (Bradford, 1976), with bovine γ globlin as the standard. The value of enzyme activity, normalized by the amount of protein, was represented as specific activity (U/mg protein).

Isolation and Analysis of Total RNA

F9 cells were plated at 0.75×10^6 cells/100 mm-diameter culture dish. After 24 hr, the medium was changed to either a normal medium or a differentiation medium. Cells were harvested at various times. Total RNA was prepared from various cells and a 129-strain mouse kidney using the TRIZOL reagent. Total RNA (15 μg) was denatured at 60°C for 15 min in 50% formamide, 20 mM 3-(N-morpholino) propanesulphonic acid, 5 mM sodium acetate, 1 mM EDTA, 6.4% formaldehyde, and then rapidly cooled on ice. Samples were separated by electrophoresis in a 0.8% denatured agarose gel, and then transferred to a Biodyne A membrane. The filter was crosslinked by ultraviolet irradiation, and prehybridized at 42°C for 16 hr in 50% formamide, 5× SSC, 5× Denhardt's, and 0.5% SDS containing denatured salmon sperm DNA (200 μg/ml) and then hybridized at 42°C for 32 hr in the above solution with 1×10^6 cpm/ml of probe cDNA. This preparation was washed twice at 60°C for 30 min with 0.1× SSC and 0.1% SDS, air-dried, and exposed to a FUJIX imaging plate, followed by analysis with a FUJI BAS1500 system.

Reverse Transcription-Polymerase Chain Reaction and Probe cDNAs

Reverse transcription was performed at 37°C for 1 hr in a 50-μl reaction mixture that contained 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.2 mM dNTPs, 2 mM DTT, 20 U of RNase inhibitor, 200 U of SuperscriptII, 1 μg of mouse kidney total RNA, and 0.1 μg of random hexamer. A polymerase chain reaction (PCR) was then performed in a 50 μl reaction mixture that contained 10 mM Tris-HCl, pH 8.3, 100 mM KCl, 3 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of PCR primers, 2.5 μl of the reverse transcription reaction as a template, and 2.5 U of rTaq DNA polymerase. The PCR was conducted at 94°C for 3 min, followed by 30 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 60 sec. For the ALPase probe, 5′-TCATCCTGCGCTGGGCC-3′ and 5′-CCAGCAAGAAGAAGCCTTTG-3′ oligonucleotides (Terao and Mintz, 1987; Kishi et al., 1989; Misumi et al., 1998) and for 5′-Nase probe, 5′-CCGGGAATTCGGGAAGTACCCATTCATAGTC-3′ and 5′-CCGGGAATTCACAGCAGCCAGGTTCTCCC-3′ (Misumi et al., 1990a,b; Resta et al., 1993) oligonucleotides were used as the primers. PCR products were subcloned into the pGEM-T Easy vector. After confirmation of the nucleotide sequence, each *Eco*RI fragment was isolated and used as probes. Probe cDNAs were labeled with α-^{32}P-dCTP by use of the BcaBest DNA labeling kit.

For M_2-PKase probe, M2PKs, 5′-TCGAGGAACTC-CGCCGCCTGGCGCCCATTACCA-3′ and M2PKas, 5′-TGGTAATGGGCG-3′ oligonucleotides which contains the nucleotide sequence common to rat and human cDNAs (Noguchi et al., 1986; Tani et al., 1988), were annealed with each other and radiolabeled with α-^{32}P-dCTP by means of the Klenow reaction.

Plasmids

The mouse L/B/K-ALPase gene 1A promoter/firefly luciferase reporter construct (1838ALP/Luc) was a gift of Dr. Tatsuya Kobayashi (Harvard Hospital) (Kobayashi et al., 1998). Mouse tail (129-strain) DNA was a gift of Dr. Tamio Noguchi (Nagoya University). The region between 5.5 and 5.0 kb upstream of the mouse L/B/K-type ALPase gene was amplified by PCR (Escalante-Alcalde et al., 1996). The PCR was performed in a 50 μl reaction mixture that contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTPs, 10 pmol of primer 5′ALP (5′-CGGGGTACC-GTCGACCAGTCACTTATTTTG-3′) and primer 3′ALP (5′-CCGGACGCGTGATCCTCTAGCAAGGCGAGC-3′), 250 ng of the 129 mouse tail DNA as a template, and 15 U of ExTaq DNA polymerase. The PCR was conducted at 94°C for 3 min, followed by 35 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min. The PCR product was ligated into the pGEM-T Easy vector. After confirmation of the nucleotide sequence, a 500 bp *Kpn*I/*Mlu*I fragment of the plasmid was subcloned into the *Kpn*I/*Mlu*I sites of the 1838ALP/Luc plasmid to produce the penhaALP/Luc. The sequences of all plasmids were confirmed by dideoxy sequencing.

DNA Transfections and Luciferase Assays

DNA transfections were carried out using the LIPOFECTAMINE PLUS reagent. All plasmids used for transfection were prepared using a QIAGEN plasmid kit, followed by CsCl gradient ultracentrifugation. Cells (5×10^4 per well) were inoculated in a 24 well plate on the day prior to transfection. Three hundred nanograms of the indicated reporter plasmid and 2 ng of the pRL-CMV plasmid were used. Three hours after transfection, the cells were treated with 5 mM NaBu or 2×10^{-7} M RA and, after 48 hr, the cells were harvested. Firefly and sea pansy luciferase assays were carried out according to the manufacturer's recommended protocol. Luciferase activities were determined by a Berthold Lumat model LB 9501. Firefly luciferase activities (relative light units) were normalized by sea pansy luciferase activities.

RESULTS

Alterations of Enzyme Activities in NaBu-Treated F9 Cells

Specific activities of ALPase, PKase, and 5′-Nase were determined for the NaBu-treated F9 cells. As shown in Figure 1A, no changes in ALPase activities were observed within 48 hr in the untreated F9 cells. In contrast, the specific activity of ALPase in the NaBu-treated F9 cells were significantly elevated at 16 hr after treatment and eventually increased by three-fold at 48 hr. This increase paralleled the morphological changes of the F9 cells to endoderm cells (data not shown). In contrast, PKase activity significantly decreased by 63% only at 48 hr after treatment (Fig. 1B). In this process, the specific activity of 5′-Nase was quite low and was not significantly changed in the F9 cells which had either treated with NaBu or had received no treatment (Fig. 1C). These findings indicate that the specific activities of these enzymes are differentially regulated during the endoderm differentiation of NaBu-treated F9 cells.

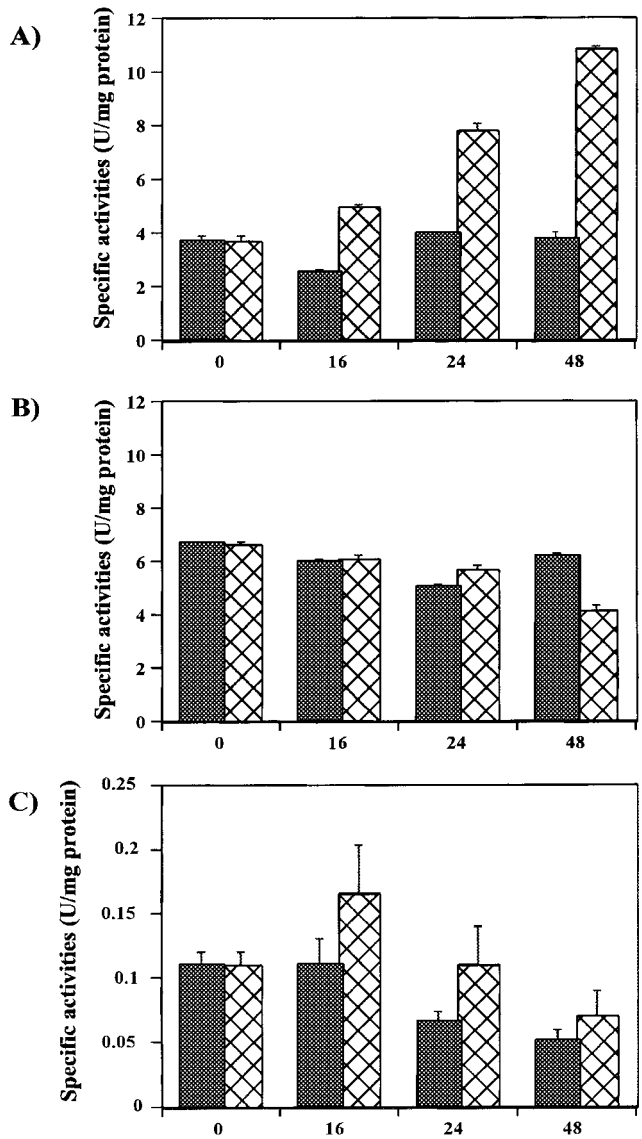

Fig. 1. Alterations in enzyme activities during endodermal differentiation of F9 cells. Specific activities of (**A**) ALPase, (**B**) PKase, and (**C**) 5′-Nase were determined at the indicated times (hr), indicated on the *bottom*. The black and hatched boxes indicate untreated and 5 mM NaBu-treated F9 cells, respectively.

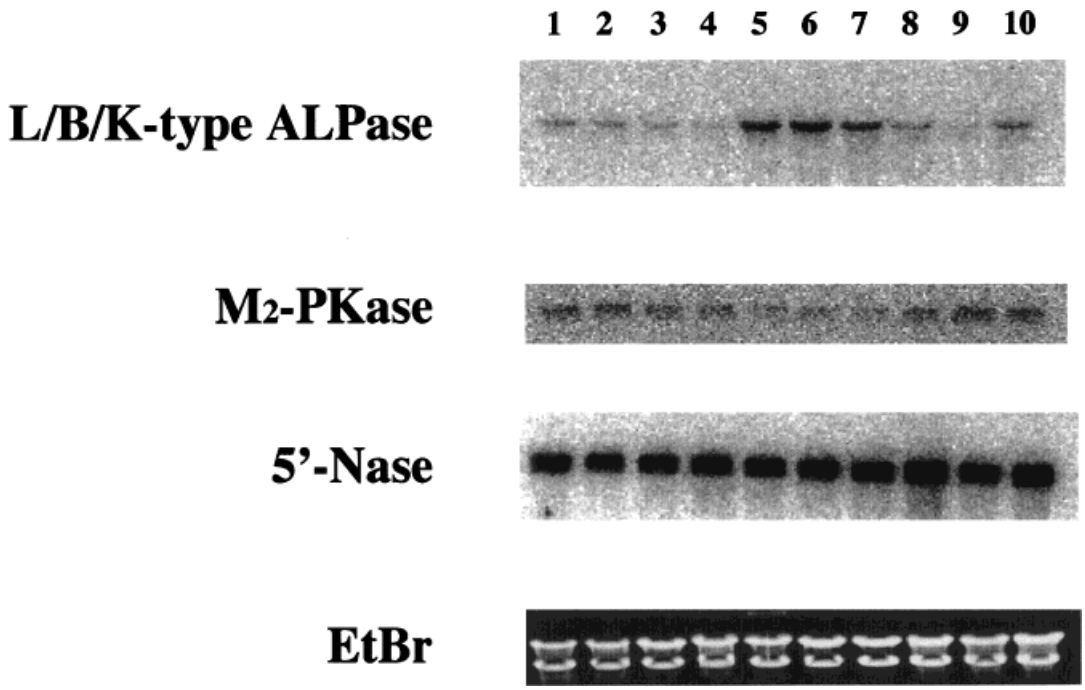

Fig. 2. Northern blot analysis of L/B/K-type ALPase, M_2-PKase, and 5′-Nase mRNAs during endoderm differentiation of F9 cells. Total RNA was prepared from F9 cells treated with or without 5 mM NaBu or 2×10^{-7}M RA. Samples of 15 μg/lane were loaded. Probe DNAs are shown on the *left*. EtBr indicates ethidium bromide staining of the gel prior to transfer to nylon filter. Lanes 1 to 4 and 9, untreated F9 cells; lanes 5 to 8, NaBu-treated F9 cells; lane 10 RA-treated F9 cells. Lane 1, just before treatment. Lanes 2 and 5, lanes 3 and 6, and lanes 4, 7, 9, and 10 indicate 24, 32, and 48 hr after treatment. Lane 8, at 32 hr after treatment with NaBu, the reagent was removed from the medium, after which, the cells were cultured for another 16 hr.

Analysis of the Levels of ALPase, PKase, 5′-Nase mRNAs During the Endoderm Differentiation of F9 Cells

To address the issue of whether alterations of specific activities of these enzymes in NaBu-treated F9 cells are dependent on quantitative alterations of the levels of their mRNAs, we next performed a Northern blot analysis. L/B/K-type ALPase, M_2-PKase, and 5′-Nase cDNAs were employed as probes.

As shown in Figure 2, untreated F9 cells showed low levels of L/B/K-type ALPase mRNA throughout the 48 hr culture period. In contrast, the level of L/B/K-type ALPase mRNA increased in both the NaBu- and RA-treated F9 cells. The level of L/B/K-type ALPase mRNA in the NaBu-treated F9 cells was elevated to 5.1-fold at 24 hr and was maintained during 48 hr of culture. At 48 hr, the mRNA level in the NaBu-treated F9 cells was higher than that in the RA-treated F9 cells. The level of M_2-PK mRNA was decreased to 44% at 24 hr in NaBu-treated F9 cells and its level was maintained up to 48 hr. In contrast, the level of M_2-PKase mRNA remained unchanged within 48 hr in the case of the RA-treated F9 cells. It has been reported that removal of NaBu from a culture medium causes the F9 cells to revert to the undifferentiated state (Kosaka et al., 1991). Interestingly, when NaBu was removed from the culture medium at 32 hr, the levels of L/B/K-type ALPase and M_2-PKase mRNAs reverted to those observed for the untreated F9 cells after 16 hr of culture. The level of 5′-Nase mRNA was not changed in all cells examined. These results indicate that alterations in the specific activity of ALPase, M_2-PKase, and 5′-Nase during the endoderm differentiation of F9 cells result from regulation of the level of their corresponding mRNAs.

Determination of Transcriptional Activity of the Mouse L/B/K-Type ALPase Gene Promoter in Differentiation-Induced F9 Cells

We then examined the issue of whether an increase in the level of L/B/K-type ALPase mRNA during the endoderm differentiation of F9 cells is based on the transcriptional activation of the mouse L/B/K-type ALPase gene. Two reporter constructs, the 1838ALP/Luc and penhaALP/Luc, were used. The former represents the region between −1,838 and +82 of the mouse L/B/K-type ALPase gene and is linked upstream of the firefly luciferase structural gene. The latter is the region between −5.5 and −5.0 kb, which is inserted upstream of the 1838ALP/Luc. These plasmids were transfected into F9 cells, which were then treated with RA or NaBu. When the 1838ALP/Luc plasmid was transfected into F9 cells and treated with RA, the relative luciferase activities were not changed (Fig. 3). In contrast, when the penhaALP/Luc plasmid was transfected, the basal relative luciferase activities were found to be 2.5-fold higher than that of the 1838ALP/Luc plasmid. In addition, when the cells were treated with RA, the relative luciferase activities increased by more than 3.4-fold. These results indicate that the

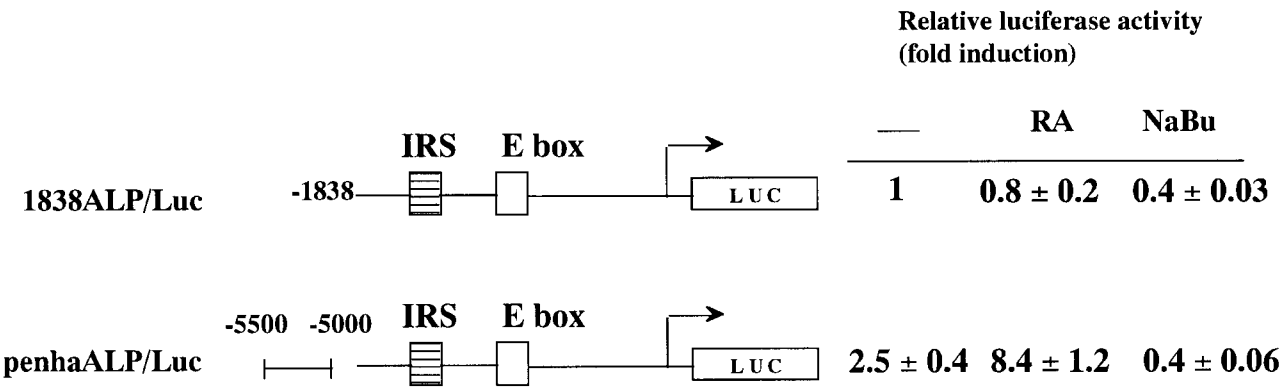

Fig. 3. Comparison of promoter activities of the mouse L/B/K-type ALPase gene in untreated and differentiation-induced F9 cells. Schematic diagrams of the reporter constructs are depicted on the *left*. The 1838ALP/Luc and penhaALP/Luc are used as reporter plasmids. Relative luciferase activities in untreated (indicated by −), RA−, and NaBu-treated F9 cells are shown on the *right*. Relative luciferase activity from the 1838ALP/Luc plasmid in untreated F9 cells is set at 1. All values shown represent the mean ± SE of at least three separate experiments.

region between −5.5. and −5.0 kb of the mouse L/B/K-type ALPase gene contains a transcriptional regulatory element for basal expression and that the region also confers RA responsiveness. Finally, we examined the issue of whether an increase of the level of L/B/K-type ALPase mRNA in NaBu-treated F9 cells occurs via the same mechanism as that observed in RA-treated F9 cells. These constructs were transfected into F9 cells, which were then treated with NaBu. Surprisingly, their relative luciferase activities were decreased to about 40% of the that of the 1838ALP/Luc, which had been transfected into untreated F9 cells.

DISCUSSION

The findings herein show that the specific activity of ALPase is elevated during NaBu-induced endoderm differentiation of F9 cells and that its induction results in an increase in the level of L/B/K-type ALPase mRNA. This event occurred in parallel with morphological changes of F9 cells to endoderm cells and expression of the laminin B1 chain mRNA as a endoderm-specific marker molecule (data not shown). We previously reported that the inner core cells of mouse OTT-6050 ascites teratoma embryoid bodies (EBs) showed a high level of ALPase activity (Matsuzawa et al., 1988). When the EBs were cultured in vitro, the specific activity of ALPase increased (Matsuzawa et al., 1988). In this case, the outer endoderm-like cells of the EBs, in addition to the inner core cells, showed a new ALPase activity, as evidenced by histological analysis. In addition, the specific activity of ALPase increased during the RA-induced endoderm differentiation of F9 cells (Yamada et al., 1992). All these observations were compatible with histochemical evidence for cells of the endoderm layer of 7.5-day mouse embryos in utero (Matsuzawa et al., 1988). The elevation of specific activities of ALPase in both the NaBu- and the RA-treated F9 cells resulted from an increase in the level of L/B/K-type ALPase mRNA. In contrast, both the specific activity of PKase and the level of M_2-PKase mRNA decreased in NaBu-treated F9 cells. A decrease in the level of M_2-PKase mRNA was not observed in the RA-treated F9 cells for a period of up to 48 hr. It has been reported that the endoderm differentiation of F9 cells by treatment with NaBu progresses faster than that by treatment with RA (Kosaka et al., 1991). Izumi et al. (1995) reported on the molecular cloning of mouse M_2-PKase cDNA as a molecule which is preferentially expressed in undifferentiated mouse embryonal carcinoma P19 cells and that the level of M_2-PKase mRNA decreased in P19 cells which had been treated with RA for 96 hr. Therefore, by treatment with RA for more longer period of time, the level of M_2-PKase mRNA could decrease, even in F9 cells. It has been also reported that the expression of M_2-PK mRNA is controlled by cell growth and differentiation (Yamada and Noguchi, 1999b). Interestingly, when NaBu was withdrawn from the medium at 32 hr after treatment, this induced a reversion of F9 cells to undifferentiated cells for a further 16 hr, and the levels of L/B/K-type ALPase and M_2-PKase mRNAs reverted to the level of the untreated F9 cells. Under the same conditions, the disappearance of the endoderm-specific molecules and a reversion in the level of c-myc mRNA was observed (Kosaka et al., 1991). Thus, it is likely that stimulation of ALPase gene expression coincides with the endoderm differentiation of F9 cells or that M_2-PKase gene expression is up-regulated by cell growth. On the other hand, the specific activity of 5′-Nase and the level of its mRNA were not altered in any of the cells. In most studies, β-actin or GAPDH mRNAs have been used as internal controls in Northern blot analysis. However, under some conditions, such as treatment with hormones or growth factors, the expression of these mRNA is not reliably in steady state. Thus, 5′-Nase mRNA represents a good candidate for an internal control, since its level was not altered during cell growth and differentiation.

Molecular cloning of the mouse L/B/K-type ALPase gene and the characterization of its E1A promoter region has been reported (Terao et al., 1990; Escalante-Alcalde et al., 1996; Kobayashi et al., 1998). Both an E box at −234 bp and an inverted repeat sequence at −1,212 bp upstream of the E1A promoter are required for basal transcription (Kobayashi et al., 1998). It has been also reported that a region between −5.5 and −5.0 kb is required for RA responsiveness (Escalante-Alcalde et al., 1996). Our findings indicate that the region confers, not only RA responsiveness, but basal expression as well. We then hypothesized that an increase in the level of L/B/K-type ALPase mRNA in NaBu-treated F9 cells is also dependent on the transcriptional activation of the mouse L/B/K-type ALPase gene promoter. If this is the case, it is then possible to examine whether the mechanism is identical with one by treatment with RA, i.e., if the NaBu-responsive element is identical with the RA-responsive element (RARE). However, our results clearly showed that reporter luciferase activities were decreased in the case of the NaBu-treated F9 cells. Several possible explanations can be proposed to explain the results. First, an increase in the level of L/B/K-type ALPase mRNA is not dependent on gene transcription but, rather, the enhancement of its stability. Second, the region examined does not confer NaBu responsiveness. Third, the transfection experiment in NaBu-treated F9 cells was not suitable for these experiments. It has been reported that cells treated with 5 mM NaBu exhibited an alteration in transfection efficiency (Gorman et al., 1983). Indeed, when the ALPase reporter plasmids were transfected into F9 cells and treated with NaBu, both firefly luciferase and sea pansy luciferase activities also markedly increased (data not shown). In addition, even when the pGL3-Control reporter plasmid, which contains SV40 enhancer/promoter linked to the firefly luciferase gene, was transfected into F9 cells and treated with NaBu, both firefly luciferase and sea pansy luciferase activities also increased but the relative luciferase activities after normalization decreased (data not shown). Therefore, it is likely that the transfection experiments in NaBu-treated F9 cells are not suitable for analyzing promoter activity of the mouse L/B/K-type ALPase gene. To address the issue of whether an increase of L/B/K-type ALPase mRNA in NaBu-treated F9 cells results in transcriptional activation of the L/B/K-type ALPase gene promoter or enhancement of mRNA stability, nuclear run-on assays and in vitro transcription assays will be required. In addition, further studies will be required for the identification of bona fide RARE of the mouse L/B/K-type ALPase gene and an analysis of a mechanism decreasing the level of M_2-PKase mRNA.

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