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Expression of ornithine carbamoyltransferase gene in rat hepatoma-derived cell lines, H4-II-E and R-Y121B

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Summary. The rat R-Y121B cell line is a unique cell line which has ornithine carbamoyltransferase (OCT, EC 2.1.3.3) and can be continuously cultured in a serum-free medium which lacks arginine but is supplemented with ornithine. The OCT gene expression was examined in R-Y121B cells and their parental H4-II-E cells. OCT activity in R-Y121B cells was about one-tenth of that of the adult rat liver while it was not detected at all in H4-II-E cells. Southern hybridization using an OCT cDNA probe containing the entire coding region showed that the OCT gene structure was apparently not different in R-Y121B cells, H4-II-E cells, or rat liver cells. Northern hybridization using the OCT cDNA probe detected a hybridizing signal in R-Y121B cells but not in H4-II-E cells. Reverse transcription-polymerase chain reaction (RT-PCR) showed that an expected size of the OCT cDNA fragment was amplified in R-Y121B cells but not in H4-II-E cells. The amplified fragment was confirmed to be a real rat OCT cDNA by the digestion of this fragment with two restriction enzymes and by the nucleotide sequencing of the fragment. These results indicated that OCT mRNA was present in R-Y121B cells but not in H4-II-E cells. Amino acid analysis showed that arginine was not present in the culture medium but was present in the hydrolysate of R-Y121B cells. The present experiments indicate that transcription, translation, and processing of OCT proceeds normally and the resultant OCT functions in R-Y121B cells, whereas the transcription does not occur in parental H4-II-E cells even though these cells have the normal gene.

Keywords: Amino acids – Ornithine carbamoyltransferase – Rat hepatoma cell line – Southern hybridization – Northern hybridization – PCR – Enzyme activity

Introduction

Mammalian cells are cultured in a medium containing amino acids, vitamins, glucose, inorganic salts, and serum. Most cells require at least 13 amino acids

for their continuous growth (Eagle, 1959; Levintow and Eagle, 1961). However, some of these amino acids are not essential amino acids for the growth of animals. Arginine is one of these non-essential amino acids. It is synthesized via the urea cycle in the liver of the animals. However, even hepatic cells require arginine for growth when transferred to *in vitro* culture. A loss of ornithine carbamoyltransferase (OCT, EC 2.1.3.3) is considered to be the major reason for the requirement of arginine (Schimke, 1964). If OCT is lost, the urea cycle does not proceed any more and subsequently arginine is not produced, which causes the arginine requirement.

OCT as the second member of the urea cycle catalyzes the production of citrulline from ornithine and carbamoylphosphate. This enzyme is mainly present in the liver of higher animals. The gene encoding OCT is localized on the X chromosome (Ricciuti et al., 1976). It is quite a large gene and spans about 75 kilobase pairs (kbp) comprised of 10 exons (Takiguchi et al., 1987; Hata et al., 1988). OCT mRNA is about 1.8kb long. OCT is synthesized on cytosolic polysomes as precursors containing amino-terminal extension. The precursors are transported into mitochondria and cleaved there to the mature size. Rat OCT consists of 322 amino acid residues and the molecular mass is 36, 135 Da (Horwich et al., 1984; Takiguchi et al., 1984; McIntyre et al., 1985; Kraus et al., 1985).

No cell line could be continuously cultured in an arginine-free medium. However, Niwa et al. (1979, 1980) have established a cell line (R-Y121B) from a rat hepatoma-derived cell line (H4-II-E) by cultivating the parental cells stepwise in a medium whose arginine was gradually replaced by ornithine. Since then, R-Y121B cells have been proliferated in a serum-free Eagle's minimum essential medium (MEM) which lacks arginine but is supplemented with ornithine. They have a population doubling time of approximately 48 hours and have been routinely subcultured in this medium. R-Y121B cells have OCT activity which is not detected in parental H4-II-E cells. However, the difference between R-Y121B cells and H4-II-E cells is not clearly understood. Therefore, in this study, we have examined the OCT gene expression in R-Y121B cells and compared it with that in parental H4-II-E cells.

Materials and methods

Cells and culture media

H4-II-E cells (Pitot et al., 1964) from a Reuber H-35 minimal deviation hepatoma (Reuber, 1961) have been maintained in our laboratory as previously reported (Niwa et al., 1980). R-Y121B cells were cultured in serum-free EM Y121B medium. The EM Y121B medium is a modified Eagle's minimum essential medium (MEM, Eagle, 1959) which lacks arginine, glutamine, and tyrosine in original Eagle's MEM but is supplemented with ornithine (0.6mM) and 5-bromodeoxyuridine (50μg/ml) (Niwa et al. 1980). The experimental medium was prepared and sterilized by autoclaving at 121°C for 15min in this laboratory. Streptomycin and penicillin were added to the media at a concentration of 100μg/ml and 100 units/ml, respectively. Culture medium was renewed every 3 or 4 days. The cells were subcultured at 1 to 2-week intervals.

Measurement of OCT activity

The cells proliferating in glass bottles were harvested with a silicone-rubber policeman and washed with phosphate buffered saline (PBS) by centrifugation. The cell pellet was stored at −20°C until assay. The cell pellet was thawed by the addition of a small volume of water and homogenized using a Physcotron homogenizer (Niti-On, Funabashi, Chiba). The liver was removed from a Sprague-Dawley rat and homogenized similarly. OCT activity in the homogenates was measured according to the method of De Gregorio (1993) and Boyde and Rahmatullah (1980). The protein content of the homogenates was measured by the method of Lowry et al. (1951). OCT activity was expressed as amounts of citrulline produced per minute per mg protein.

Amplification of OCT cDNA by PCR

A sense primer F (5′-CTGTCTAATTTGAGGATCCTG-3′) and an antisense primer R (5′-GAACTTTGGCTTCTGGAGCAC-3) corresponding to the 5′ end (nucleotides 4–24) and 3′ end (nucleotides 1042–1062) of OCT cDNA, respectively (Takiguchi et al., 1984; McIntyre et al., 1985), were synthesized with a DNA synthesizer (Model 392, Applied Biosystems, Foster City, CA). Total RNA was extracted from the H4-II-E and R-Y121B cells and the first strand of cDNA was synthesized with an oligo(dT) primer and reverse transcriptase using the Superscript Preamplification System (BRL, Gaithersburg, MD). The OCT cDNA was amplified by PCR in a 100 μl reaction mixture containing 4 μl of the first strand of cDNA solution, 0.2 μM each of the F and R primers, and 2.5 units of AmpliTaq DNA polymerase using a Gene Amp PCR Reagent Kit (Perkin-Elmer Cetus, Norwalk, CT). After an initial melting at 94°C for 1 min, 30 cycles of PCR (melting: 94°C, 1 min; annealing: 55°C, 1 min; extension: 72°C, 2 min) were carried out using Zymoreactor II (Atto, Tokyo). A final extension was done at 72°C for 10 min and the solution was kept at 4°C. A portion of the PCR product was electrophoresed on a 1% agarose gel and photographed under ultraviolet illumination.

Whether or not the DNA fragment amplified by the PCR was real OCT cDNA was examined by the digestion of the fragment with the restriction enzymes. Ten μl of the PCR product were mixed with 0.3 μl of *Kpn*I (0.3 units, Nippon Gene, Tokyo). Another 10 μl were mixed with *Xho*I (2.2 units, Nippon Gene). The mixtures were each incubated at 37°C for 2 h. The whole reaction mixtures were electrophoresed on a 2% agarose gel.

A portion of the PCR product was electrophoresed on a 1% low-melting-temperature agarose gel. A gel slice containing about 1-kb fragment of OCT cDNA was cut out under ultraviolet illumination. The DNA fragment was purified according to the standard method (Sambrook et al., 1989), radiolabelled, and used as a probe for hybridization as described below.

Sequencing of the PCR product

The rest of the aforementioned PCR product above was purified with a Microcon-100 concentrator (Amicon, Beverly, MA). The nucleotide sequence of the PCR product was determined by use of a DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). The F and R primers were used as the sequencing primers. After an initial melting at 94°C for 1 min, 50 cycles of PCR (melting: 94°C, 1 min; annealing: 55°C, 1 min; extension 72°C, 4 min) were carried out. The solution was kept at 4°C. After the reaction, the reaction product was extracted with phenol/chloroform, precipitated with ethanol, and dried according to the method specified by the supplier of the reagent. It was then dissolved in a loading solution and subjected to a DNA sequencer (Model 373A, Applied Biosystems).

Labeling of probe DNA

The OCT cDNA fragment amplified by the PCR was labeled with [α-^{32}P]dCTP (~110TBq/mmol, Amersham, Backinghamshire) by the method of Feinberg and Vogelstein (1983) using a Random Primer DNA Labeling Kit (Takara, Kyoto). A rat β-actin cDNA fragment was obtained from Clontech (Palo Alto, CA) and was labeled in the same way.

Southern hybridization

High molecular weight DNA was extracted from the rat liver, H4-II-E cells and R-Y121B cells according to the standard method (Sambrook et al., 1989). The DNAs (10μg) were digested with 20 units of *Eco*RI, *Bam*HI or *Hin*dIII (Nippon Gene) and electrophoresed on 0.9% agarose gel. The DNA fragments were transferred to a nylon membrane (GeneScreen Plus, NEN Research Products, Boston, MA) according to the method of Southern (1975). The membrane was hybridized with the [^{32}P]-labeled OCT cDNA probe. Hybridization and washing of the membrane were carried out as described before (Konno et al., 1995). The membrane was wrapped with a plastic film and exposed to an imaging plate. The plate was then analyzed with an image analyzer (Model BAS 2000II, Fuji Photo Film, Tokyo).

Northern hybridization

Total RNA was extracted from H4-II-E and R-Y121B cells by the method of Chomczynski and Sacchi (1987) using the Isogen solution (Nippon Gene, Tokyo). The RNAs (15μg) were electrophoresed on a 1.2% denaturing agarose gel. The nucleic acid was transferred to a nylon membrane (Hybond N, Amersham). The membrane was hybridized with the [^{32}P]-labeled OCT cDNA probe at 65°C for approximately 16hr. The membrane was washed twice with 2 × SSPE/0.1% SDS for 10min each at room temperature. [1 × SSPE is a solution of 0.13M NaCl/0.01M sodium phosphate/1mM EDTA (pH 7.7).] The membrane was washed 1 × SSPE/0.1% SDS at 65°C for 15min and washed twice in 0.1 × SSPE/0.1% SDS at 65°C for 10min each. The membrane was wrapped with a plastic film and exposed to an imaging plate, which was analyzed with an image analyzer.

The OCT cDNA probe was removed from the membrane in a boiling solution of 0.1% SDS. Then, the membrane was rehybridized with the [^{32}P]-labeled rat β-actin probe as described above.

Amino acid analysis

R-Y121B cells were cultured in the EM Y121B medium for three days. A small volume of the culture medium was taken out and filtrated through a ultrafiltration membrane (a molecular cut-off value of 10,000, Ultrafree C3GC, Millipore). The filtrate was examined for the amino acid concentration with a conventional amino acid analyzer (Model L8500, Hitachi, Tokyo). The remaining cells were harvested by a silicone-rubber policeman and precipitated by centrifugation. The precipitated cells were washed twice with PBS by centrifugation. They were resuspended in distilled water and homogenized. The homogenate was hydrolyzed with 6 N hydrochloric acid and examined for the amino acid concentration with the amino acid analyzer.

Results

OCT activity was examined in R-Y121B cells, their parental H4-II-E cells and the rat liver homogenate (Table 1). H4-II-E cells did not have OCT activity whereas R-Y121B cells had OCT activity. The enzyme activity in R-Y121B cells was about 1/10 of that of the rat liver homogenate. These results are consistent with the previous reports (Niwa et al., 1979, 1980).

Table 1. OCT activity in cell lines and rat liver

	Activity (μmol/min·mg protein)*
H4-II-E	not detectable (4)
R-Y121B	0.09 ± 0.01 (4)
Rat liver	1.06 ± 0.08 (4)

*The enzyme activity is expressed as the mean ± standard deviation. The number of samples used for the assay is shown in parentheses.

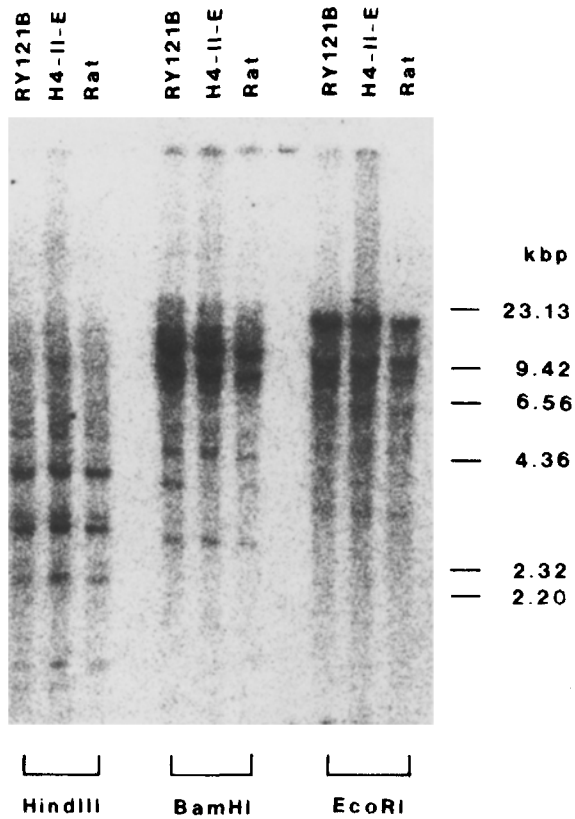

Fig. 1. Southern hybridization. DNA was extracted from R-Y121B cells, H4-II-E cells and the rat liver. It was digested with *Hin*dIII, *Bam*HI or *Eco*RI. The DNA fragments were electrophoresed, transferred to membranes and hybridized with the OCT cDNA containing the entire coding region

DNA was extracted from R-Y121B cells, H4-II-E cells and the rat liver, and was digested with *Eco*RI, *Bam*HI or *Hin*dIII. The DNA fragments were electrophoresed, transferred to a membrane and hybridized with the OCT cDNA probe containing the entire coding region. A large number of hybridizing bands were observed (Fig. 1). However, the hybridization patterns were almost the same among R-Y121B cells, H4-II-E cells and the rat liver. These results indicate that the structure of the OCT gene in R-Y121B cells and H4-II-E cells is not significantly different from that of the rat hepatic cells.

Total RNA was extracted from the R-Y121B cells and H4-II-E cells, electrophoresed, transferred to a membrane and hybridized with the OCT cDNA probe. A hybridization signal was detected in R-Y121B cells but not in H4-II-E cells (Fig. 2A). When the probe was removed and the membrane was re-hybridized with a control β-actin probe, both cells gave hybridization signals with similar intensity (Fig. 2B), indicating that similar amounts of RNA were present on the lanes of R-Y121B and H4-II-E cells. Therefore, these results indicate that the message for OCT is present in R-Y121B cells but not in H4-II-E cells.

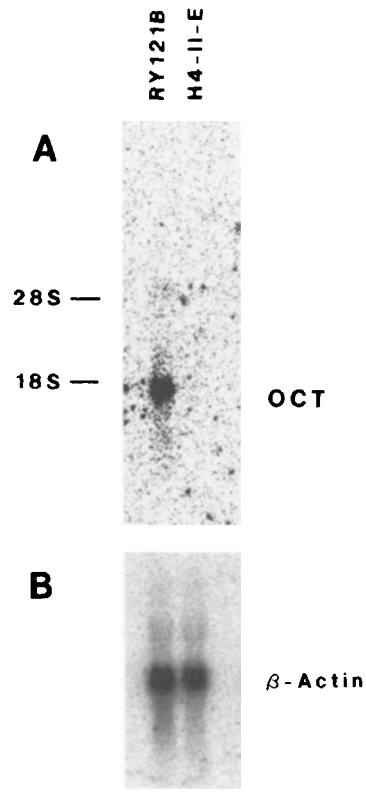

Fig. 2. Northern hybridization. Total RNA was extracted from R-Y121B cells and H4-II-E cells. It was electrophoresed, transferred to the membrane and hybridized with the OCT probe (the upper panel: **A**). After the OCT probe was removed from the membrane, the membrane was re-hybridized with a control β-actin probe (the lower panel: **B**)

Total RNA was extracted from R-Y121B and H4-II-E cells and was reverse transcribed into cDNA. OCT cDNA was amplified by PCR using oligonucleotide primers specific to the OCT cDNA (Fig. 3A). Figure 3B shows that a DNA fragment of the expected size was amplified in R-Y121B cells but not in H4-II-E cells. The PCR product was found to be cleaved into two fragments by the digestion with *Xho*I or *Kpn*I (data not shown) as expected from the nucleotide sequence of the OCT cDNA (Fig. 3A). Furthermore, when the amplified fragment was partially sequenced, it contained the sequences corresponding to the nucleotides 31–257 and nucleotides 791–970 (Fig. 4) of the rat liver OCT cDNA (Takiguchi et al., 1984; McIntyre et al., 1985). These results clearly indicate that the amplified fragment is OCT cDNA. Therefore, the results of the reverse transcription-PCR confirm that OCT mRNA is present in R-Y121B cells but not in H4-II-E cells.

R-Y121B cells were continuously cultured in the serum-free EM Y121B medium which lacked arginine but contained ornithine. After they were grown in the fresh EM Y121B medium for three days, a small volume of the culture medium was taken out and filtrated through an ultrafiltration mem-

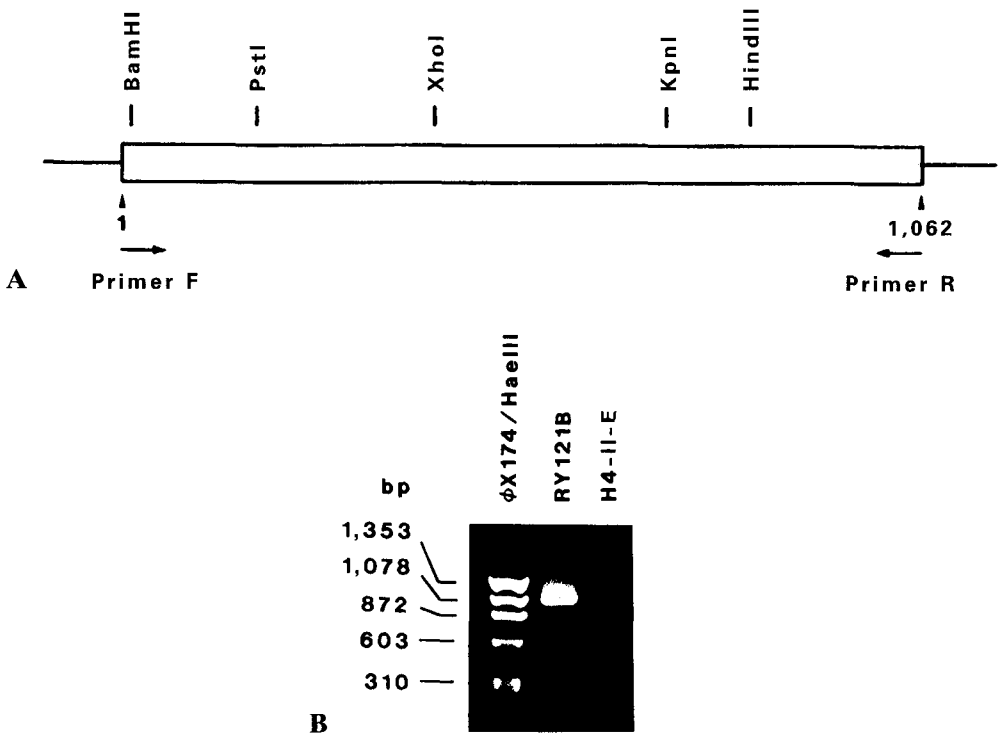

Fig. 3. PCR amplification of OCT cDNA. The upper panel (**A**) shows the structure of OCT cDNA and the primers used for PCR. The bar shows the coding region of OCT cDNA. The restriction sites are shown above the bar. The lower panel (**B**) shows the electrophoretic pattern of the PCR products. Total RNA was extracted from R-Y121B and H4-II-E cells and was reverse transcribed. The resultant cDNA was used as a template for PCR together with the primer F and R. The PCR products were electrophoresed on an agarose gel together with the size markers (ϕX174 DNA digested with *Hae*III)

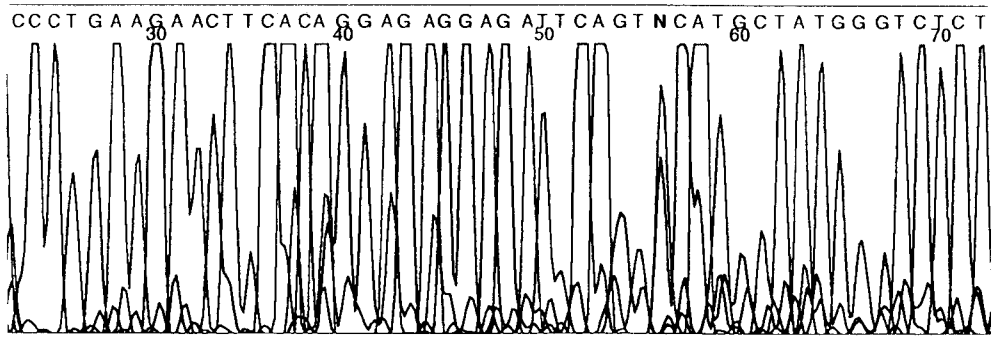

Fig. 4. Partial nucleotide sequence of the DNA fragment amplified by PCR. Total RNA was extracted from R-Y121B cells was reverse transcribed. OCT cDNA was amplified by PCR and the amplified DNA fragment was partially sequenced. The nucleotide sequence shown corresponds to nucleotides 131–174 of rat OCT cDNA

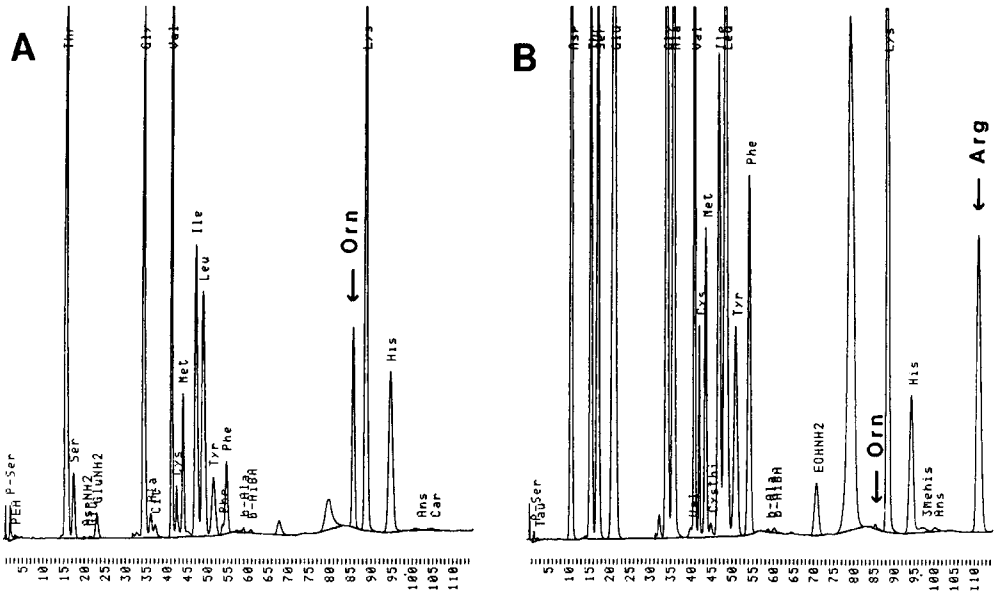

Fig. 5. Amino acid analysis of culture medium and cell hydrolysate. R-Y121B cells were grown for three days in the serum-free EM Y121B medium which lacked arginine but was supplemented with ornithine. A small volume of the medium was filtrated through an ultrafiltration membrane (molecular cut-off value of 10,000) and examined for amino acid concentration (the left panel: **A**). The remaining R-Y121B cells were washed with PBS and hydrolyzed with HCl. The hydrolysate was then examined for amino acid concentration (the right panel: **B**). Note that arginine was not present in the medium (**A**) but was present in the cell hydrolysate (**B**), and that ornithine, on the contrary, was present in the medium (**A**) but was present only very slightly in the cell hydrolysate (**B**)

brane (a molecular cut-off value of 10,000). The filtrate was examined for amino acid concentration with an amino acid analyzer. The remaining cells were harvested and washed with PBS. The cell pellet was then resuspended in distilled water and hydrolyzed with HCl. The hydrolysate was also subjected to the amino acid analysis. Figure 5 shows that arginine was not present in the culture medium (Fig. 5A) but was present in the hydrolysate of R-Y121B cells (Fig. 5B). Ornithine was present abundantly in the medium (Fig. 5A) but present only very slightly in the cell hydrolysate (Fig. 5B). These results suggest that R-Y121B cells synthesized the necessary arginine via the urea cycle from ornithine which was supplemented in the medium. This result implies that OCT is functioning normally in R-Y121B cells.

Discussion

The R-Y121B cell line was established from the H4-II-E cell line using a selective culture medium which favored the growth of the cells having OCT activity (Niwa et al., 1979, 1980). The present experiment has shown that R-Y121B cells and H4-II-E cells have the OCT gene which is not different from that of rat liver cells (Fig. 1). This gene is transcribed in R-Y121B cells whereas it is not in H4-II-E cells (Figs. 2 and 3). Consequently, R-Y121B cells have OCT activity whereas H4-II-E cells do not (Table 1). The OCT is functioning normally in R-Y121B cells because their cellular proteins contains a significant amount of arginine (Fig. 5).

The OCT activity in R-Y121B cells was about 10% of that of the rat liver homogenate. The activity was almost the same as that determined about 16 years ago by Niwa et al. (1979, 1980). R-Y121B cells have been cultured continuously during this period. Therefore, this level may be the maximum level attainable by culturing the cells in the medium which lacks arginine but is supplemented with ornithine. Widman et al. (1979) observed that rat hepatocyte-hepatoma cell hybrids had 6–10% of the OCT activity of rat liver extract. Delers et al. (1984) also observed that variant cells obtained from H4-II-EC3 possessed not more than 7.5% of the OCT activity of normal hepatocytes when they were cultured in a medium in which arginine was replaced by ornithine. This level of OCT activity may be sufficient to sustain the continuous growth of the cells in an arginine-free, ornithine-supplemented medium.

Rat OCT gene is a fairly large gene spanning about 75 kbp. It is cleaved by *Hin*dIII into 23 fragments of various sizes (Takiguchi, 1987). Human and mouse OCT genes have structures similar to that of the rat OCT gene. Human OCT gene is cleaved into 26 fragments by *Eco*RI and 10 fragments by *Bam*HI (Hata et al., 1988). The digestion of DNAs from rat liver, H4-II-E cells, and R-Y121B cells by *Hin*dIII, *Eco*RI and *Bam*HI produced many fragments hybridized with the OCT probe (Fig. 1). Therefore, these results are consistent with the above reports.

The OCT message was present in R-Y121B cells but not in H4-II-E cells (Fig. 2). The size was a little smaller than 18S ribosomal RNA, indicating the

size is about 1.8 kb. This size is quite consistent with the reported size for OCT mRNA in the rat liver (Takiguchi et al., 1984; Kraus et al., 1985).

R-Y121B cells have mRNA for OCT and resultant OCT activity whereas their parental H4-II-E cells do not. Therefore, it may be possible to reveal what kind of factor(s) is necessary for the expression of the OCT gene by examining R-Y121B cells and H4-II-E cells. Widman et al. (1979) and Farmer and Goss (1991) found that the cell fusion between the hepatoma cells and normal hepatocytes was necessary for the activation of the silent OCT gene in the hepatoma cells. Farmer and Goss (1991) postulated that some activator for the OTC expression was lacking in the hepatoma cells. Delers et al. (1984) found that 5-azacytidine treatment induced the OCT expression in the rat hepatoma cells and suggested that hypermethylation was important for this induction. Nishiyori et al. (1994) showed the hepatocyte nuclear factor-4 and CCAAT/enhancer binding protein were necessary for the specific expression of this gene in the liver. It would therefore be interesting to determine whether or not these factors are also functioning in R-Y121B cells.

Disorder in OCT is known to cause a severe hyperammonemia in humans. Since R-Y121B is the unique cell line which express OTC and is continuously cultured in a very limited nutritional environment, it would be a useful cell line for the study of the function and regulation of OCT *in vitro*.

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