Cca3, the mRNA level of which transiently decreases before initiation of DNA synthesis in regenerating rat liver cells

Yasuyuki Hayashi[a,*], Masutaro Ichinose[b], Hirofumi Yuasa[b], Masae Tatematsu[b], Masahide Ishibashi[a]

[a] *Laboratories of Viral Oncology, Research Institute, Aichi Cancer Center, Chikusa-ku, Nagoya 464, Japan*
[b] *Laboratory of Pathology, Research Institute, Aichi Cancer Center, Chikusa-ku, Nagoya 464, Japan*

Received 21 January 1997; revised version received 27 February 1997

Abstract Kinetics of cca1, cca2, cca3 and rat gas1 mRNA levels were compared with those of DNA synthesis level in regenerating rat liver cells after partial hepatectomy. A transient decrease of cca3 mRNA level and an increase of rat gas1 mRNA level were observed before initiation of DNA synthesis, followed by a rapid decrease of rat gas1 mRNA level. By molecular cloning and nucleotide sequencing, cca3 cDNA was found to consist of 4514 nucleotides with a large open reading frame of 3027 nucleotides, encoding a protein of 1009 amino acids with three copies of an ankyrin repeat-like sequence.

Key words: Rat liver regeneration; cca3 cDNA cloning; Ankyrin repeat

1. Introduction

Accumulating evidence indicates that cell growth is controlled by the balance between growth-promoting and growth-suppressing genes. Although many growth-promoting genes have been isolated, a relatively few with growth-suppressive function have been obtained thus far. To facilitate understanding of the cell-growth control, more attention needs to be focused on this area. One approach is to study genes the mRNAs of which are preferentially accumulated in growth-arrested cells. Thus a growth arrest-specific 1 (gas1) cDNA has been isolated by subtractive hybridization between mRNAs extracted from growing mouse cultured line cells and cDNAs derived from the serum-starved cells [1]. Prohibitin cDNA was similarly isolated on the basis of preferential accumulation of the corresponding mRNAs in the normal rat liver cells but not in the regenerating counterparts [2]. Both cDNAs are confirmed to play a role in cell-growth suppression [3,4].

We previously isolated three Confluent 3Y1 Cell-Associated (cca1, cca2 and cca3) cDNAs and a rat homologue of gas1 cDNA [3] on the basis of preferential accumulation of the corresponding mRNAs in growth-arrested rat 3Y1 line cells (will be published elsewhere). To address the question whether accumulation of three cca mRNAs and rat gas1 mRNA was associated with the growth-arrested cell state in vivo, kinetics of the four mRNA levels were compared with those of DNA synthesis level in regenerating rat liver cells after partial hepatectomy (PH). As a result, the significant alternations of cca3 and rat gas1 mRNA levels were observed during the early stage of rat liver regeneration. Isolation of cca3 cDNA and characteristics of the corresponding protein are described.

*Corresponding author. Present address: Department of Gene Research, Cancer Institute, JFCR, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan. Fax: (81) 3-5394-3902

Abbreviations: aa, amino acid; cca, confluent 3Y1 cell-associated; gas, growth arrest-specific; nt, nucleotide; ORF, open reading frame; PH, partial hepatectomy; RACE, rapid amplification of the cDNA ends; UTR, untranslated DNA region

The sequence data for cca3 in this paper have been submitted to the GenBank/EMBL/DDBJ Data libraries under accession number AB000216.

2. Materials and methods

2.1. Cells

COS cells were cultured in Dulbecco's modified Eagle's minimal essential medium with 10% fetal calf serum and then used for transient expression of cca3 cDNA. The cca3 cDNA expression vector prepared as described below was introduced into the cells by electroporation (Gene Pulser, BIO-RAD) at 250 V, 975 μF.

2.2. Molecular cloning and nucleotide sequencing of cca3 cDNA

The cca3 cDNA fragment (171 nucleotides (nts)) used as a probe DNA for cca3 cDNA cloning was obtained as one of four cDNA clones whose corresponding mRNAs were preferentially accumulated in confluent rat 3Y1 line cells but not in their sub-confluent counterparts (will be published elsewhere).

A cDNA library containing 2×10^6 recombinant phages was constructed with 1 mg of poly(A)$^+$ RNA extracted from confluent 3Y1 cells (SuperScript Choice system, BRL). For screening, 1×10^5 plaques of the amplified library were plated at a density of 5000 plaque forming units/100×150 mm^2 dish. Duplicate lifts were made from each of 20 dishes using nylon membranes (Gene Screen, DUPONT). The membranes were then hybridized with the cca3 probe labeled with [^{32}P]dCTP, and the plaques that specifically hybridized with the probe were isolated. After a second plaque hybridization, two clones were isolated. The longer clone was found to contain a 3 kb cDNA region. On rescreening the library, a cDNA larger than 3 kb was not isolated. Therefore, the remaining 5′-DNA region (1.5 kb) was generated using the 5′-RACE (rapid amplification of cDNA ends) method (5′-AmpliFINDER RACE, CLONTECH). Three independent clones were isolated from independent PCR, and the sequence was determined (Sequencing PRO, TOYOBO). The DNA fragment obtained was ligated, at *Xho*I, to the above cDNA clone and the obtained DNA fragment was used as the cca3 cDNA (4514 nts). To amplify the entire open reading frame (ORF) of 3027 nts (nt414–nt3440), the 5T4USAL primer containing cDNA region (nt366–nt393) and the 5T4TSAL primer corresponding to the cDNA sequence (nt3431–nt3463) were used.

2.3. Plasmid construction

The cca3 cDNA was cloned into the pMOS vector (Amersham) so as to obtain pMOS-5T4C. A pMOS-cca3(-atg) covering a cca3 cDNA region of 3058 nts (nt417–nt3474) was constructed from the pMOS-5T4C, by replacing the six nucleotides (nt411–nt416) containing the first ATG site of the cDNA with a *Sal*I site and deleting the 5′-untranslated DNA region (UTR; nt1–nt413) and almost all of the 3′-UTR (nt3475–nt4514). To express cca3 cDNA in COS cells at high

PII S0014-5793(97)00258-5

level, the pEF-BOS vector containing the promoter region of human elongation factor 1α and SV40 replication origin was used [5]. After removing the stuffer DNA by digesting the vector with *Bst*XI and *Not*I, the DNA sequence for the Myc epitope (10 aa; EQKLISEEDL) located just before the new cloning sites (*Bam*HI, *Sal*I or *Kpn*I) was introduced as a *Bst*XI–*Not*I fragment so as to produce the expression vector for a Myc epitope-tagged protein (pEF-Myc). pEF-Myc cca3 containing the cDNA for the deduced Myc-tagged CCA3 protein (amino acid (aa)2–aa1009) was constructed by cloning the *Sal*I–*Kpn*I fragment of pMOS-cca3(-atg) into *Sal*I and *Kpn*I sites of the pEF-Myc vector.

The rat gapdh sequence was amplified with specific primers (CLONTECH) by PCR, using oligo-dT primed 3Y1 cDNAs as template DNAs. The PCR product was cloned into the pMOSblue-T vector (Amersham) and then the resulting plasmid, pMOS-gapdh, was used for preparation of a control probe in RNase protection assay described below.

2.4. Animals and treatment

Fischer rats (10 weeks old, male) were subjected to PH and triplicate rats were killed at the indicated times as described earlier [6]. The DNA synthesis levels in regenerating liver cells were monitored by determining the labeling indices [7]. Briefly, rats received an injection of bromodeoxyuridine (BrdU; 100 mg/kg of body weight) at 1 h before being killed and the labeling indices were determined immunohistochemically.

2.5. RNA preparation

Triplicate RNA samples of rat liver cells were prepared at each time point after PH and without PH using ISOGEN (Nippon Gene). RNA samples were also prepared from several organs of 10-week-old Fischer rats (male) for examining the cca3 mRNA level.

2.6. RNase protection assay

The RNase protection assay was performed with an antisense-strand RNA probe synthesized with T7 polymerase and [^{32}P]UTP (MAXIscript, Ambion). RNA samples were checked for equal relative amounts of RNA by staining ribosomal RNAs with ethidium bromide and hybridized with the probe at 42°C overnight. After RNase treatment with 0.5 U of RNaseA and 20 U of RNaseT1, the protected RNA was electrophoresed in 5% urea-polyacrylamide gels.

2.7. Western blot analysis

For Western blotting, cell lysates were prepared by adding a sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue), followed by vigorous mixing and boiling for 5 min. After SDS-polyacrylamide gel electrophoresis, the proteins were blotted electrophoretically onto polyvinylidene di-

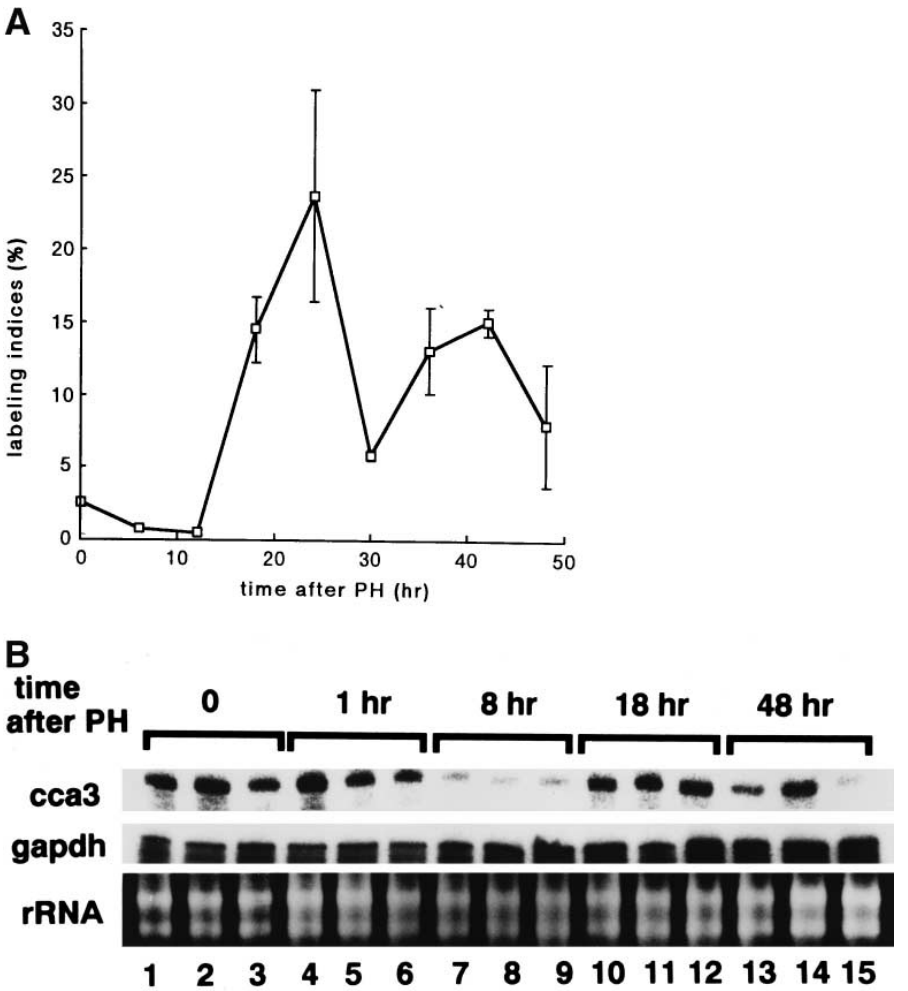

Fig. 1. Comparison of kinetics of cca3 mRNA level with those of DNA synthesis level during rat liver regeneration. A: Kinetics of DNA synthesis level during liver regeneration. Liver samples were obtained from the triplicate rats given BrdU 1 h before being killed. The DNA synthesis levels at 6, 12, 18, 24, 30, 36, 42 and 48 h after PH and time=0 (without PH) were examined by calculating the labeling indices. Open squares and bars indicate means and SDs, respectively. B: Kinetics of cca3 mRNA level during liver regeneration. Liver RNA samples were prepared from the triplicate rats at 1 h (lanes 4–6), 8 h (lanes 7–9), 18 h (lanes 10–12) or 48 h (lanes 13–15) after PH and without PH (time=0; lanes 1–3). The cca3 and gapdh mRNA levels were examined by RNase protection assay. Ribosomal RNAs (28S and 18S) are also shown.

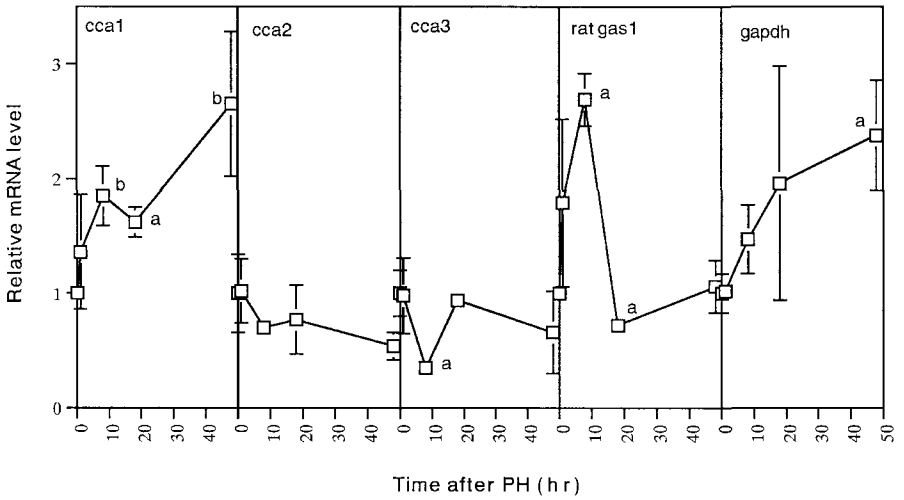

Fig. 2. Comparison of kinetics of cca3 mRNA level with those of cca1, cca2, rat gas1 and gapdh mRNA levels. The relative mRNA levels of cca1, cca2, cca3, rat gas1 and gapdh at the different time points (1, 8, 18 and 48 h after PH) as compared to time = 0 were summarized. Results are shown as means (open squares) with SDs (bars). $^{a}P < 0.01$; $^{b}P < 0.05$ versus time = 0.

fluoride membranes (MILLIPORE), which were then blocked by soaking in PBS-T buffer (0.1% Tween-20 in PBS) supplemented with 10% nonfat dry milk and 0.1% sodium azide at 4°C overnight. After washing with PBS-T buffer, the membranes were incubated with rabbit anti-Myc epitope antibodies (MBL) at 3 mg/ml for 1 h, incubated with horseradish peroxidase-labeled goat anti-rabbit IgG antibodies (Zymed) for 1 h, and then the protein detected was visualized using the ECL system (Amersham).

3. Results and discussion

Kinetics of DNA synthesis level during rat liver regeneration after PH were monitored by calculating the labeling indices (Fig. 1A). Labeling indices increased at 18 h after PH and reached the first peak at 24 h and the second peak at 42 h after PH, which was consistent with a previous observation by others [8]. An increase in the mRNA level for hepatocyte growth factor, which is thought to trigger hepatocyte growth, is observed before initiation of DNA synthesis [9]. Then, kinetics of three cca and rat gas1 mRNA levels monitored by RNase protection assay (Fig. 1B) were compared with those of DNA synthesis level as follows. The relative levels of the above four mRNAs at the different time points as compared to time=0 were summarized in Fig. 2. The cca3 mRNA level transiently decreased before initiation of DNA synthesis (8 h after PH), whereas the rat gas1 mRNA level increased at the same time point, followed by a decrease thereafter (18 h after PH). Although no significant change in the cca2 mRNA level was noted, a tendency to decrease was observed during the time course of liver regeneration (8–48 h after PH). In contrast, the cca1 mRNA level gradually increased at these time points. Similarly, the control gapdh mRNA level showed a tendency to increase after initiation of DNA synthesis. The initial mRNA levels were found to be restored 5 weeks after PH, when the liver regeneration was long completed (data not shown). Since the level of cca3 mRNA changed significantly as compared to those of the other two cca mRNAs during liver regeneration, the corresponding cca3 cDNA was isolated.

A cca3 cDNA clone (3 kb) was isolated from the library of the confluent 3Y1 cells using the cca3 cDNA probe, which was obtained by the mRNA differential display method as described in Section 2. The remaining DNA region (1.5 kb) was generated by the 5′-RACE method and then ligated to the above cDNA at the overlapping *Xho*I site (nt1849). The cca3 cDNA obtained was found to consist of 4514 nts, which was compatible with the size (4.5 kb) of the corresponding mRNA estimated by Northern blot analysis (will be published elsewhere). Downstream of the 5′-UTR (413 nts), there was a large ORF of 3027 nts, encoding a 1009 aa protein. To preclude the possibility that the ORF was a chimeric artifact between two partially identical fused ORFs, a RT-PCR experiment was performed with the 5T4USAL and 5T4TSAL primers, designed to amplify the entire ORF as described in Section 2. A single product with the expected size, having the expected restriction enzyme sites, was detected in confluent 3Y1 cells (data not shown). The DNA sequence (CCCATGG) including the first ATG (nt414) resembles Kozak's consensus sequence [10]. The 3′-UTR (1071 nts) contained a polyadenylation signal (AATAAA) at nt4492 and a consensus ATTTA element involved in mRNA instability [11] at 4319. The DNA region (171 nts) used as the cca3 probe for isolating the cca3 cDNA clone was located at nt4344 to nt4514. Homology searching for the predicted amino acid sequence indicated

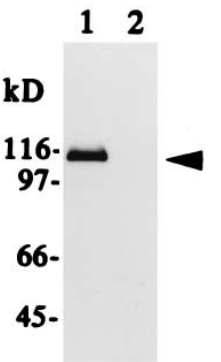

Fig. 3. Western blot analysis of CCA3 protein. Cell lysates were prepared from COS cells transfected with pEF-Myc cca3 (lane 1) or pEF-Myc vector (lane 2) and incubated with anti-Myc epitope antibodies. The location of a protein detected with the antibodies is indicated by the arrow head. The bars on the left show the locations of the molecular mass markers.

Fig. 4. Distribution of cca3 mRNA in different rat organs. RNA samples were prepared from rat brain (lane 1), spleen (lane 2), liver (lane 3), kidney (lane 4) and testis (lane 5) and examined for the cca3 mRNA level by the RNase protection assay. The gapdh probe was used as a control.

that three copies of an ankyrin repeat-like [12] sequence were located at aa519–aa552, aa566–aa598 and aa605–aa637. The identity was 36% to the eleventh repeat of the ankyrin motif (AN11) for the sequence of aa519–aa552, 39% to AN7 for aa566–aa598 and 42% to AN8 for aa566–aa598. A protein with the molecular mass of 115 kDa was detected with the anti-Myc epitope antibodies in COS cells transiently transfected with the cDNA for the deduced Myc-tagged CCA3 protein (pEF-Myc cca3) (lane 1, Fig. 3).

RNA samples from different rat organs were examined for the cca3 mRNA level. The levels of cca3 mRNA in brain, spleen, kidney and testis were comparable to that in liver (Fig. 4).

Kinetic analysis of three cca and rat gas1 mRNA levels during rat liver regeneration showed the characteristic alternations of cca3 and rat gas1 mRNA levels in the early stage (see Fig. 2), suggesting their roles in the initiation of hepatocyte growth. Indeed, mouse gas1 has been reported to exert growth suppressive effect on mouse line cells [3]. It remains to be elucidated whether the cca3 cDNA has the growth suppressive activity.

Acknowledgements: We appreciated Ms. C. Yamada for her helpful technical assistance and Dr. K. Koike for his helpful comments in preparing this manuscript. This work was supported, in part, by a Grant-in-Aid for a Special Project Program from Aichi Cancer Center to Y.H.

References

[1] C. Schneider, R.M. King, L. Philipson, Cell 54 (1988) 787–793.
[2] J.K. McClung, D.B. Danner, D.A. Stewart, J.R. Smith, E.L. Schneider, C.K. Lumpkin, R.T. Dell'Orco, M.J. Nuell, Biochem Biophys Res Commun 164 (1989) 1316–1322.
[3] G. DelSal, M.E. Ruaro, L. Philipson, C. Schneider, Cell 70 (1992) 595–607.
[4] M.J. Nuell, D.A. Stewart, L. Walker, V. Friedman, C.M. Wood, G.A. Owens, J.R. Smith, E.L. Schneider, R. Dell'Orco, C.K. Lumpkin, D.B. Danner, J.K. McClung, Mol Cell Biol 11 (1991) 1372–1381.
[5] S. Mizushima, S. Nagata, Nucl Acids Res 18 (1990) 5322.
[6] T. Imai, T. Masui, H. Nakanishi, K. Inada, K. Kobayashi, T. Nakamura, M. Tatematsu, Carcinogenesis 17 (1996) 19–24.
[7] M. Tatematsu, K. Ogawa, M. Mutai, T. Aoki, T. Hoshiya, N. Ito, Cancer Res 51 (1991) 318–323.
[8] I. Jacob, J.I. Fabrikant, J Cell Biol 36 (1968) 551–565.
[9] R. Zamegar, M.C. DeFrances, D.P. Kost, P. Lindroos, G.K. Michalopoulos, Biochem Biophys Res Commun 177 (1991) 559–565.
[10] M. Kozak, Nucl Acids Res 15 (1987) 8125–8148.
[11] G. Shaw, R. Kamen, Cell 46 (1986) 659–667.
[12] C.S. Birkenmeier, R.A. White, L.L. Peters, E.J. Hall, S.E. Lux, J.E. Barker, J Biol Chem 268 (1993) 9533–9540.