The induction of apoptosis in HeLa cells by the loss of LBP-p40

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Abstract

To analyze the function of the laminin-binding protein precursor p40 (LBP-p40) in higher eukaryotic cells, plasmid DNA expressing antisense or sense cDNA for p40 under the control of the LacSwitch system was introduced into HeLa cells. Stable transformants were isolated, and the expression of p40 was assayed by Western and Northern blotting. The expression level of p40 was not affected in HeLa cell transformants cultured in 10% serum-supplemented media with the induction of antisense (AS)-p40 with 5 mM IPTG. However, both the protein and message for endogenous p40 in serum-depleted media with 5 mM IPTG were reduced to about 30–10% of the expression level in serum-free media without 5 mM IPTG. Colony formation was inhibited with the suppression of p40. AS-p40 clones died in 7 days when cultured in serum-depleted media with 5 mM IPTG, while clones without 5 mM IPTG AS-p40 clones never died, even in serum-depleted media. Additionally, sense (S)-p40 clones and control CAT clones survived more than 2 weeks in serum-free media with 5 mM IPTG. DNA fragmentation assay revealed that cell death induced by the reduction of AS-p40 resulted from apoptosis. Both the inhibition of cell growth and apoptotic cell death were partially rescued by the transfer of the p40 cDNA expression vector to AS-p40 clones. Moreover, the introduction of a synthetic hammerhead ribozyme for LBP-p40 using a fusogenic viral liposome suppressed the message for LBP-p40 even in the presence of 10% serum, and it also induced apoptosis.

Keywords: LBP-p40; antisense RNA; ribozyme; cell-growth; apoptosis

Abbreviations: LBP-p40, laminin-binding protein precursor 40 kilo-dalton protein; AS, antisense; S, sense; IPTG, isopropyl- β -D-thiogalactoside; CAT, chloramphenicol acetyl transferase

Introduction

A cDNA for the 40 kDa protein was first cloned as an abundant protein in cancer cells (Yow *et al*, 1988; Makrides *et al*, 1988). The DNA sequence corresponded to the 67 kDa laminin-binding protein first isolated from cell extracts by laminin-affinity chromatography (Rao *et al*, 1983; Lesot *et al*, 1983; Malinoff *et al*, 1983; Wewer *et al*, 1986) and was extensively investigated as a protein responsible for tumor metastasis (Liotta *et al*, 1986; Sobel, 1993). Therefore, it was identified as a precursor molecule of the laminin-binding protein, p40 (LBP-p40) (Yow *et al*, 1988). However, Northern blot analysis using p40 cDNA as a probe showed a single band of approximately 1.1–1.2 kb (Yow *et al*, 1988; Makrides *et al*, 1988; van den Ouweland *et al*, 1989). A recent report indicated LBP-p67 might be a dimer of LBP-p40 and that acylation might occur before or after dimerization (Landowski *et al*, 1995). Overproduction of LBP-p40 in BHK cells increases the production of the 67 kDa protein at the cell surface (Wang *et al*, 1992). Although there is no direct evidence, it has been proposed that p40 may be a cytoplasmic precursor for LBP-p67. However, the nomenclature of the laminin-binding protein precursor generated some confusion with progress in the analysis of p40 since LBP-p40 appeared to exhibit multiple functions in various aspects of cell growth, embryonic development, and cancer progression. Several lines of evidence have indicated that the p40 is localized exclusively in the cytoplasm and is a component of 40S ribosomal subunits in mammalian, *Arabidopsis*, and *Urechis caupo* cells (Auth and Brawerman, 1992; Garcia-Hernandez *et al*, 1994; Tohgo *et al*, 1994; Rosenthal and Wordeman, 1995). It has also been proposed that p40 is detected in developing mice retina and participates in defining the dorsal/ventral axis in developing retinas (Rabacchi *et al*, 1990; McCaffery *et al*, 1990). Wang *et al* reported LBP-p40 to be the Sindbis virus receptor (Wang *et al*, 1992). Several mutations in *Drosophila* p40 resulted in a zygotic lethal by affecting oogenesis and imaginal disc development (Melnick *et al*, 1993). Together, these observations indicate that p40 expression is correlated with cell growth. Recently, YST1 and YST2 homologous to human LBP-p40 were cloned in *Saccharomyces cerevisiae*, which has no laminin (Ellis *et al*, 1994). The disruption of either YST1 or YST2 resulted in slow growth, probably due to reduced levels of the 40S ribosome, while the complete knock-out of the YSTs was lethal (Demianova *et al*, 1996). This suggests that Yst proteins might be required for translation and that they contribute to assembly and/or stability of the 40S ribosomal subunit, raising the questions of whether or not the reduction of LBP-p40 would affect the growth of somatic cells or higher eukaryotes and, if the reduction is lethal, what the mode of cell death would be.

We isolated a monoclonal antibody which recognized 40 kDa protein in the nuclear envelope, cytoplasmic

particles and perichromosomal region (Wataya-Kaneda *et al*, 1987), and further analysis of the antigen (Kaneda *et al*, 1993) indicated that the protein was associated with both chromatin DNA and nuclear matrix, and from these results we suspected that the molecule might be required for anchoring chromatin DNA to the nuclear matrix. Recently, we isolated the antigen from the cytoplasmic particles and identified it as LBP-p40. Then, we confirmed that epitope-tagged LBP-p40 could be sorted to the nucleus as well as ribosome, and that the LBP-p40 was tightly attached with the nuclear matrix (Sato *et al*, 1996). A series of our investigations of LBP-p40 suggests that LBP-p40 may play some important role in the nucleus such as the stabilization of chromatin DNA.

No direct evidence has been presented on the function of LBP-p40, since it has not been successful to regulate the expression level of p40 in higher eukaryotic cells.

In this study, we used the LacSwitch inducible mammalian expression system (Stratagene Inc.) with lactose operon (Bourdeau *et al*, 1995; McCarthy *et al*, 1995) for the analysis of p40 gene function in mammalian cells. Stable transformants were established in HeLa cells to induce sense- or antisense-p40. Induction of antisense p40 resulted in apoptotic cell death in a serum-depleted condition in which the expression of endogenous p40 was reduced to 10–30% of the expression level without induction. DNA fragmentation assay showed that the suppression of p40 accelerated apoptotic cell death in a serum-free condition. Transient expression of exogenous p40 partially rescued apoptotic cell death. Moreover, the introduction of a synthetic ribozyme for LBP-p40 suppressed the expression of LBP-p40, even in serum-supplemented media, and induced DNA fragmentation.

Results

p40 expression in inducible antisense (AS) or sense (S)-clones

We first examined CAT gene expression in pOPI3 CAT transfectants of lacI-clone No. 15 cultured in the presence and absence of IPTG. We detected the CAT gene transcript in five out of 22 clones. Induction of the CAT message was varied. In clone No. 8 (control clone), the CAT message level induced by IPTG was highest among the all the message tested. The message was detected at 2 h after the addition of 5 mM IPTG, reached its peak level between 4 and 24 h and then gradually decreased (Figure 1A). The CAT message induced by IPTG was not altered by serum-depletion, and 5 mM IPTG affected neither cell growth nor total protein, RNA, or DNA synthesis (data not shown).

Next, we examined the p40 mRNA by Northern blotting using p40 cDNA as a probe. When stable transformants of AS-p40 or S-p40 were cultured in 10% FCS-DMEM, the p40 message was not changed by IPTG (Figure 1B). We previously observed that p40 expression was suppressed in slow-growing cells, in cells in a confluent state, and in cells cultured in serum-depleted media, and that to the contrary, p40 expression was enhanced in cancer cells, in cells in a subconfluent state, and in cells cultured in serum-

Figure 1 Gene expression in HeLa cell transformants analyzed by Northern blot. (A) CAT gene transcript induced by 5 mM IPTG was assayed by Northern blot analysis at 0, 2, 4, 24, 48 and 72 h after IPTG treatment. (B) Cells were cultured in 10% FCS-DMEM with IPTG (lanes 2, 4) or without IPTG (lanes 1, 3) for 5 days. Total RNA was isolated from S-p40 clone No. 24 (lanes 1, 2), and AS-p40 clone No. 11 (lanes 3, 4) and approximately 10 μ g total RNA of each clone was subjected to formalin gel electrophoresis, transferred to Hybond N⁺ membrane (Amersham), and hybridized with a ³²P-labeled p40 or GAPDH cDNA probe. (C) Total RNA was isolated from HeLa cell clone No. 15 expressing lacI (lanes 1, 2), clone No. 8 expressing CAT (lanes 3, 4), S-p40 clone No. 18 (lanes 5, 6), S-p40 clone No. 25 (lanes 7, 8), AS-p40 clone No. 16 (lanes 9, 10) and AS-p40 clone No. 11 (lanes 11, 12). Cells were treated with 5 mM IPTG (lanes 1, 3, 5, 7, 9, 11) or without IPTG (lanes 2, 4, 6, 8, 10, 12) in serum-depleted media for 6 days. Northern blot analysis was performed. (D) Total RNA was isolated from AS-p40 clone No. 11 on day 0 (lane 1), and on day 6 in serum-free media with IPTG (lane 2). Lane 3 shows the p40 transcript on day 9 in serum-free media when IPTG was removed from the culture on day 5 and the culture was continued for another 4 days in serum-depleted media

rich media. Consistent with this finding, p40 transcript in the present study was detected in cells cultured in serum-free media with and without IPTG. In a serum-free condition, p40 level in non-transfectant HeLa, or lacI-clone No. 15 and clone No. 8 expressing inducible CAT was reduced to 20–30% of each cell in 10% FCS-DMEM (data not shown). In AS-clone Nos. 11 and 16, p40 was suppressed by IPTG treatment compared with cells in media without IPTG (Figure 1C). A significant reduction (more than 50%) of p40 mRNA was observed in three out of 40 AS-clones (Nos. 11, 16 and 33). In S-p40 clones, the p40 message was either unchanged (38 out of 40 clones) or slightly induced (two out of 40 clones) by IPTG. Especially, in AS-clone No. 16, the p40 message was reduced to approximately 10% measured by an image analyzer

(BASS 2000, Fuji). The effective clones contained a NotI insert for p40 cDNA detected by Southern blot (data not shown). Moreover, when IPTG was removed from the culture of AS-clone No. 11 on day 5 following the IPTG treatment in serum-free media, by day 9 the p40 expression level was recovered on preIPTG treatment level (Figure 1D).

Next, the p40 protein level was analyzed by Western blotting using anti-p40 polyclonal antibody. The amount of p40 was not significantly affected by the addition of 5 mM IPTG when cultured in 10% FCS media (data not shown). However, p40 was greatly suppressed in AS-clone, Nos. 11 and 16 by the treatment of IPTG when cultured in serum-free media, whereas it was not reduced in the CAT transferred HeLa clone (Figure 2). By the densitometrical assay, the ratio of the intensity for LBP-p40 signal in the presence of IPTG to that in the absence of IPTG was about 93%, 26% and 12% in CAT alone No. 8, AS-p40 clone No. 11 and No. 16, respectively. Although the anti-p40 polyclonal antibody recognized the 67 kDa protein (LBPP67; arrowhead B) as well as the 40 kDa protein (p40; arrowhead A), the expression of LBPP67 was almost identical among clones even in the presence of IPTG. The band (approximately 44 kDa) pointed by an arrowhead C was not specific for anti-LBP-p40 antibody, since it was also detected by pre-immune rabbit serum.

Viability of AS- and S-p40 transformants

As LBP-p40 has been considered an essential protein for cellular growth, we investigated cell proliferation of AS- and S-p40 clones by labeling viable cells with tetrazolium salts. As shown in Figure 3, the growth of AS-clones decreased day by day with IPTG-treatment in serum-depleted media, while the growth of both S-clones and control clones did not decrease significantly. Additionally, when IPTG was removed on day 3

from AS-clone No.11, cellular growth was not suppressed. It is suggested that cell-growth is correlated with p40 expression. For further analysis of this phenomenon, we assayed colony formation in various clones. As shown in Table 1, in AS-clone No. 16, the number of colonies formed in serum-free media with 5 mM IPTG was less than 10% of that without IPTG. In both the control clone and the S-p40 clone, under the same culture conditions, the colony formation rate exceeded 80% of that without IPTG. Even in the presence of 5 mM IPTG, all the clones were alive when cultured in 10% FCS media (data not shown). In AS-clone No. 10, where the p40 mRNA was reduced by 30–40% with the IPTG treatment, the colony formation in serum-free media with IPTG was 58.4% of that without IPTG. The viability of cells appeared to be correlated with the decrease of the p40 transcript.

To examine this possibility directly, the p40 expression vector was transferred into AS-No. 11 using lipofection. As a control, the LacZ gene expression vector was also transferred to AS-clone No. 11. Seven days after transfection, the colony formation of those clones was assayed as described above. The rate of colony formation was raised from 26–56% with the transfer of p40 cDNA, while by the transfer of the LacZ gene, the rate was not significantly changed when cultured in serum-free media, with or without IPTG (Table 2). This procedure was repeated three times and similar results were obtained each time. Moreover, in AS-clone Nos. 16 and 33, colony formation was also enhanced by the transfer of the p40 cDNA expression vector (data not shown).

Apoptosis in AS-p40 clones

To analyze whether the IPTG-induced death of AS-p40 clones resulted from apoptosis, we used digoxigenin-labeled

Figure 2 Western blot analysis of p40 expression in each transformant. Whole cell extract was isolated from HeLa cell clone No. 8 expressing CAT (lanes 1, 2), AS-p40 clone No. 11 (lanes 3, 4), and AS-p40 clone No. 16 (lanes 5, 6). Cells were treated with 5 mM IPTG (lanes 2, 4 and 6) or without IPTG (lanes 1, 3 and 5) in serum-free media for 6 days before preparation of cell extract. Approximately 75 µg protein was separated in SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blotted with anti-p40 rabbit polyclonal antibody followed by peroxidase-labeled anti-rabbit IgG. The arrow and large arrowhead indicate LBP-p40 and LBP-p67, respectively. The small arrowhead shows a band also detected by preimmune serum

Figure 3 Cell proliferation analysis by colorimetric assay. Cells (1000) were inoculated into a 96-well plate and cultured in serum-free DMEM with or without 5 mM IPTG for 5, 6, and 7 days. At each time point, cells were labeled with MTT-[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]. The absorbance at 590 nm of MTT formazan produced from active cells was measured using a multiwell spectrophotometer. The ordinate means the percentage of absorbance at 590 nm in the presence of IPTG to that in the absence of IPTG. The abscissa shows the culture period of cells in serum-depleted condition. HeLa, non-transfectant (—■—), S-p40 clone No. 18 (—●—), AS-p40 clone No. 11 (—▲—), No. 16 (—◆—) and No. 33 (—□—). (—○—) indicates AS-p40 clone No. 11 when 5 mM IPTG was added to the culture in serum-free media for the initial 3 days, then removed, and the culture was continued

Table 1 Colony formation of HeLa cells and AS-p40 clones on day 8 after culturing in serum-free DMEM with or without 5 mM IPTG

Cell	Colony number		Ratio (b)/(a) %
	(a) IPTG (–)	(b) IPTG (+)	
HeLa, non-transfectant	426	368	86.4
HeLa-CAT No. 8	326	262	80.4
Sense-p40 No. 18	388	376	96.9
Anti sense-p40 No. 10	332	194	58.4
Anti sense-p40 No. 11	374	69	18.4
Anti sense-p40 No. 16	368	30	8.2

Colonies of more than 30 cells were counted after Giemsa staining. Mean colony number is presented

Table 2 Colony formation of AS-p40 clone No. 11 7 days after the transfer of p40 or LacZ gene in serum-depleted condition

Transferred gene	Colony number	
	IPTG (–)	IPTG (+)
pEBc-p40	186 (212, 160)	104 (97, 111)
pEBc-LacZ	216 (238, 194)	56 (65, 47)

Colonies of more than 30 cells counted after Giemsa staining. Mean colony number is presented with the actual number of colonies shown in parenthesis

genomic DNA with Apoptag (Figure 4), to detect apoptotic cells. Approximately 10% of the AS-No. 11 clones were positively stained with anti-digoxigenin fluorescein conjugate on day 6 after culturing in serum-free DMEM with 5 mM IPTG. The rate for Apoptag-stained cells was 5–30% in the other AS clones, Nos. 16 and 33 cultured in serum-free DMEM with 5 mM IPTG (data not shown). The rate for the control No. 8 clone and the S-18 clone stained using the Apoptag assay kit on day 6 in serum-free DMEM with 5 mM IPTG was 1–3%. When cells were cultured in serum-free DMEM without IPTG, less than 1% of the cells in each clone were apoptotic. When the media contained 10% FCS–DMEM, less than 0.1% of cells were stained by Apoptag, and the percentage was not affected by IPTG. Counter-staining by Hoechst 33258 showed that the nuclei of apoptotic cells were shrunk and condensed.

To detect the rate of cellular DNA fragmentation, BrdU-labeled DNA fragments in a cytoplasmic fraction were counted using an ELISA after culturing in serum-free DMEM with or without IPTG. Fragmented DNA increased 3.4-fold in AS-clone No. 16 in the presence of 5 mM IPTG, compared with cells in the absence of IPTG (Table 3). In AS-clone No. 11, DNA fragmentation was slightly increased by the addition of 5 mM IPTG. DNA fragmentation was greatly inhibited by the gene transfer of pEBc p40 to AS-p40 No. 16, but not by the transfer of pEBc-LacZ. In both the control clone No. 8 and the S-clone No. 18, DNA fragmentation was not significantly changed by IPTG treatment.

These results indicate that the loss of p40 induced apoptotic cell death. However, in the LacSwiTh inducible system, growth inhibition was not observed except when the AS-p40 clones were cultured in serum-free media with IPTG. We needed to determine whether the apoptotic cell death by the loss of p40 would be specific for the serum-depleted condition.

Effect of the ribozyme for LBP-p40

We suppressed the expression of LBP-p40 by transfer of the ribozyme for LBP-p40. Using the fusogenic viral-cationic liposome, an FITC-labeled wild-type ribozyme was introduced into 100% of the HeLa cells; fluorescence was exclusively present in the cytoplasm, and faded out in 3–5 days. We synthesized three different 38 mer ribozymes for LBP-p40, all of which digested a 29-mer targeted sequence of LBP-p40 *in vitro* (data not shown). However, when introduced into the HeLa cells by the liposome, one ribozyme, No. 111, recognized the GUC sequence of LBP-p40 mRNA at Nos. 220 to 222 nucleotides was the most effective for suppressing the LBP-p40 message. By the use of this ribozyme, the transcript for LBP-p40 was decreased day by day even in the media supplemented with 10% FCS and reduced to less than 10% of cells treated with HIV-Tat ribozyme on day 4 and 6, while the human GAPDH transcript was not significantly reduced by No. 111 ribozyme (Figure 5A). The digested RNA by the ribozyme was not detected in the HeLa cell extract using Northern blot, although those ribozyme cleaved synthesized p40 substrates into two fragments when mixed in a tube (data not shown). By the introduction of LBP-p40 ribozyme No. 111, fragmented DNA of HeLa cells cultured in 10% FCS–DMEM increased between day 4 and 6, while DNA fragmentation was not induced with the control Tat ribozyme (Figure 5B). We evaluated apoptosis by counting nick-end labeled cells (Figure 5C). With the introduction of LBP-p40 ribozyme No. 111, of more than 500 cells observed, the number of nick-end labeled cells was approximately 15% on day 4 and gradually increased to 22% on day 6. Neither the Tat ribozyme nor the empty HVJ-cationic liposome generated more than 3% nick-end labeled cells.

Discussion

This study shows that apoptotic cell death is correlated with the reduction of p40, and that the transient expression of exogenous p40 partially rescued this phenomenon. This finding seems to be consistent with previous reports on LBP-p40 indicating the relationship between p40 and cellular growth (Makrides *et al*, 1988; Rabacchi *et al*, 1990; McCaffery *et al*, 1990; Melnick *et al*, 1993; Ellis *et al*, 1994). However, no previous reports have documented apoptotic cell death by the suppression of p40. In this respect, our induction of apoptosis in AS-p40 clones may indicate a novel function of LBP-p40 in higher eukaryotic cells.

In our first trial, the expression of p40 was suppressed by the induction of antisense-p40 by 5 mM IPTG in serum-free media. Therefore, we deliberately controlled for the influences of IPTG and serum withdrawal to show the effect of the loss of p40. CAT-expressing clones under the control of IPTG in serum-free media neither inhibited growth nor induced apoptosis, and 5 mM IPTG was not toxic to cells either in a serum-free or serum-rich condition. Among AS-p40 clones, the inhibition of colony formation was well correlated with the rate of p40 suppression. Finally, by the transfer of exogenous p40 driven by a CMV promotor, both colony formation and apoptosis were partially recovered. From these results, we conclude that both the inhibition of

cell-viability and the induction of apoptosis resulted from the loss of p40, not from IPTG treatment in serum depletion. The expression of endogenous p40 was reduced to 20–30% in a serum-depleted condition

compared with the level in serum-supplemented media. The change of p40 expression by S-or As-p40 was not detected unless serum was depleted. In other words, the expression of p40 in serum-rich media was too abundant to

Figure 4 Apoptosis in each transformant. Apoptotic cells in HeLa-CAT clone No. 8 (**A**, **B**) or AS-p40 clone No. 11 (**C**, **D**) were detected by anti-digoxigenin fluorescein conjugate (Apoptag) on day 6 after culturing cells in serum-depleted media with (**B**, **D**) or without (**A**, **C**) 5 mM IPTG. Nuclei are stained with Hoechst 33258. Focus is adjusted on the condensed nuclei

Table 3 Apoptosis evaluated by cellular DNA fragmentation ELISA

Cell	Absorbance at 450 nm	
	IPTG (–)	IPTG (+)
HeLa-CAT No. 8	0.064 (0.010)	0.069 (0.019)
Sense-p40 No. 18	0.081 (0.016)	0.081 (0.020)
Anti sense-p40 No. 11	0.068 (0.006)	0.084 (0.003)
Anti sense-p40 No. 16	0.065 (0.003)	0.223 (0.014)
Anti sense-p40 No. 16+pEBc-p40	0.070 (0.005)	0.118 (0.007)
Anti sense-p40 No. 16+pEBc-LacZ	0.068 (0.006)	0.215 (0.006)

Cells labeled with BrdU were cultured in serum-free media with or without IPTG, and fragmented DNA in the cytoplasm was measured by the absorbance at 450 nm on day 6 after labeling. Values in parentheses indicate standard deviation. AS-p40 No. 16+pEBc-p40 (or pEBc-LacZ) means AS-p40 clone No. 16 transfected with p40 expression vector, pEBc-p40 (or LacZ expression vector, pEBc-LacZ) by lipofection 1 day before IPTG treatment

be decreased by AS RNA. However, the ribozyme was more powerful for reduction of the targeted gene expression. Using our fusogenic viral liposome, ribozymes as well as AS-oligodeoxynucleotides can be directly introduced into the cytoplasm without degradation (Kitajima *et al*, 1997). It is estimated that more than a half-million molecules of oligonucleotides can be introduced into one cell by this delivery system (Dzau *et al*, 1996). Therefore, both the suppression of p40 and DNA fragmentation were achieved by the ribozyme delivery even in serum-supplemented condition. This also confirms apoptosis was induced by the loss of p40.

Although no direct evidence has yet been elucidated, LBP-p40 is considered to be a precursor of LBP-p67 (Landowski *et al*, 1995). Western blotting showed that p40 was decreased by the induction of AS-p40, while the antisense did not affect the expression of p67 (Figure 2). Although this does not give us direct evidence of the relationship between these two molecules, it is unlikely that apoptotic cell death resulted from the expression level of LBP-p67.

The mechanism of apoptotic cell death by the loss of p40 remains to be elucidated. It has been suggested that LBP-p40 is required for translation in plants (Garcia-Hernandez *et al*, 1994), lower eukaryotes (Rosenthal and Wordeman, 1995; Demianova *et al*, 1996) and presumably in higher eukaryotes (Auth and Brawerman, 1992; Tohgo *et al*, 1994). The rate of protein synthesis in AS-p40 clones was assayed by ³H-leucine incorporation. However, no significant reduction was observed in AS-p40 clones compared with that in control CAT clones (data not shown). Therefore, it is unlikely that apoptosis was induced by the suppression of net protein synthesis. It may be supposed that p40 is required for the translation of some specific molecules. When the expression of bcl-2 in AS-p40 clones was analyzed by Western blotting, the expression level of bcl-2 was almost comparable with that in control CAT clones (data not shown). As described in Introduction section, we reached LBP-p40 from the analysis of chromosomal proteins, and have anticipated that LBP-p40 might be an anchoring protein to stabilize chromatin DNA in the nucleus (Wataya-Kaneda *et al*, 1987; Kaneda *et al*, 1993; Sato *et al*, 1996). Recently, we discovered that

recombinant LBP=p40 tightly bound to DNA cellulose column through the association with core histones (unpublished observation), and we also succeeded in co-precipitation of nuclear matrix proteins with LBP-p40. We recently found that AS-p40 clones were arrested at the S phase of the cell cycle (unpublished data). Therefore, taken together, we speculate that chromatin DNA may become unstable by the loss of LBP-p40 as a chromatin anchoring protein and that the unstable chromatin DNA might be liable to nuclease attack during cell-cycle arrest. Although much more extensive studies are required to elucidate the relationship between apoptosis by the loss of LBP-p40 and S-phase arrest, since LBP-p40 is an essential protein in higher eukaryotic cells, the reduction by the ribozyme of LBP-p40 in cancers may lead to a new approach for cancer therapy.

Materials and Methods

Plasmid DNA construction

The LacSwitch inducible mammalian expression system (Stratagene Inc.) consisted of p3'SS containing a lacI gene and pOPI3CAT containing a lac operator. Human LBP-p40 cDNA, isolated from a HeLa cell cDNA library, kindly provided by Dr H Nojima, Osaka University, and NotI linker was ligated at 5' and 3' termini of the cDNA. The pOPI3 sense or antisense-p40 contained right- or reverse-oriented entire sequence for p40 cDNA at NotI sites of the pOPI3CAT plasmid, respectively. All plasmid DNAs were purified by cesium chloride ultracentrifugation.

Isolation of transformants

We transferred 20 µg of p3'SS to 10⁶ HeLa cells by the calcium-phosphate precipitation method, and cloned 30 stable transformants after a 2-week selection by hygromycin B (300 µg/ml). The nuclear expression of lacI was examined by both protein blotting and indirect immunofluorescence using rabbit polyclonal antibody against recombinant lacI (Statagene Inc.). Of the clones isolated, clone No. 15 expressed the highest level of lacI protein, and all the No. 15 cells showed clear nuclear staining by indirect immunofluorescence using the anti-lacI antibody. Using a calcium-phosphate precipitation method, 20 µg of pOPI3 sense or antisense-p40 was transferred to 10⁶ cells of clone No. 15. As a control, pOPI CAT was transferred to cells of clone No. 15 by the same method. Stable transformants were cloned by the selection of 900 µg/ml geneticin (G418, Gibco BRL) in each case.

Cell culture

HeLa cells were maintained in Dulbecco's modified Eagle minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). To induce sense or antisense-p40 in the LacSwitch induction system, cells were cultured in 10% FCS-DMEM or serum-depleted DMEM containing 5 mM IPTG. Fresh medium with or without IPTG was supplemented every 2 days.

Northern and Western blotting

Total RNA from each clone was isolated using Isogen (Nippon Gene, Inc.), and an equal amount of the RNA (8–15 µg) was separated in

formalin gel and subjected to Northern blotting using 32 P-labeled cDNA for LBP-p40 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Clontech) or chloramphenicol acetyltransferase (CAT) as a

probe. The intensity of each signal was measured by an image analyzer (BASS 2000, Fuji). For protein analysis, cells subconfluent in a 60 mm dish were harvested and lysed in modified RIPA lysis buffer

Figure 5 Effects of the ribozyme for LBP-p40. **(A)** The transcript for LBP-p40 was detected by Northern blot analysis. Total RNA of HeLa cells treated with fusigenic viral-cationic liposomes containing HIV-TAT ribozyme (lanes 1, 2 and 3) or LBP-p40 ribozyme No. 111 (lanes 4, 5 and 6) was subjected to Northern blot analysis on day 2 (lanes 1 and 4), day 4 (lanes 2 and 5), and day 6 (lanes 3 and 6) after the ribozyme transfer. Human GAPDH was also probed as an internal control. **(B)** DNA fragmentation of HeLa cells introduced with HIV-Tat ribozyme (—●—) or LBP-p40 ribozyme No. 111 (—■—) by fusigenic viral-cationic liposome was assayed on day 2, 4 and 6 after the transfer. The ordinate indicates the absorbance at 450 nm. The abscissa shows the period of cells cultured in 10% FCS-DMEM after ribozyme transfer. **(C)** The nick-end labeled HeLa-cells detected by Apoptag (colonies of more than 500 cells) were microscopically observed on day 0, 4 and 6 after introduction of HIV-Tat ribozyme (—●—), LBP-p40 ribozyme No. 111 (—■—), or BSS without ribozyme (—▲—) by fusigenic viral-cationic liposome

(50 mM Tris-Cl pH 8, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% deoxycholate, 5 mM EDTA) containing 2 mM phenylmethylsulfonyl-fluoride. Supernatant was obtained after centrifugation (12 000 g, 10 min), and the amount of protein was measured by bicinchoninic acid assays (Pierce). Approximately 75 µg of protein was subjected to Western blotting, and the signal was detected using anti-p40 polyclonal antibody followed by a horseradish peroxidase-conjugated second antibody and visualized by chemiluminescence reagent (ECL, Amersham Inc.). The intensity of each signal was measured by a densitometer (Becton-Dickinson).

Cell viability assay

Approximately 10^4 cells were inoculated into each well of 6-well plates (Corning) and cultured for 1 day in 10% FCS-DMEM. The medium was then changed to serum-depleted DMEM with or without 5 mM IPTG. After fixing with cold 80% methanol and staining with 5% Giemsa, the number of colonies exceeding 30 cells was counted. To evaluate the cell-growing conditions, 10^3 cells inoculated into 96-well plates (Corning) were cultured in serum-free DMEM with and without 5 mM IPTG for 5, 6, and 7 days. At each time point, cells were labeled with MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). The absorbance at 590 nm of MTT formazan produced from active cells was measured using a multiwell spectrophotometer with an MTT assay kit (Boehringer). In each case, values recorded were the average of triplicate readings.

Apoptosis assay

Cells on glass slides were fixed with 4% paraformaldehyde (pH 7.4) and treated with terminal deoxynucleotidyl transferase. Apoptotic cells were visualized with an anti-digoxigenin fluorescein conjugate using an Apoptag Plus kit (Oncor). To quantify apoptosis, 3×10^3 cells were inoculated onto 96-well-plates and DNA fragmentation was assayed by ELISA using a Cellular DNA Fragmentation ELISA kit (Boehringer Mannheim). Values used were the average of triplicate assays.

Gene transfer of p40 expression vector

The full length of p40 cDNA was cloned in our original pEBc vector, which contained a cytomegalovirus promotor to drive an inserted cDNA and Epstein-Barr virus replicon apparatus (Ori P and EBNA-1 sequence) for autonomous replication of the plasmid. We transferred 8 µg of pEBc p40 into 5×10^5 cells using LipofectAmine (Gibco BRL). As a control, LacZ gene was cloned in our pEBc vector and transferred into cells as described above. Judging from the LacZ gene expression by X-gal staining, 20–40% of the cells were transiently transduced from experiment to experiment under this transfection condition, and the gene expression level did not decline for more than 1 week when our pEBc vector was used.

Ribozyme transfer

Hammerhead ribozymes for LBP-p40 were synthesized by Hitachi-Kasei Inc. (Tsukuba, Japan). We entrapped 50 µg ribozyme in liposome consisting of phosphatidylcholine, cholesterol, and 3β [N-(N',N'-dimethylaminoethane)-carbonyl]cholesterol(DC-Chol), and the liposome was fused with UV-inactivated HVJ (Hemagglutinating Virus of Japan; Sendai virus) to form the fusigenic viral-cationic liposome (Saeki *et al*; manuscript submitted), which was 10–100 times more effective in the transfer of genes to cultured cells than our former fusigenic viral anionic liposome (Dzau *et al*, 1996). HeLa cells were

incubated with the liposome at 37°C to introduce the ribozyme. Using the fusigenic viral-cationic liposome, fluorescence-labeled ribozyme was introduced into the cytoplasm of all the HeLa cells (unpublished observation). The ribozyme for the Tat protein of human immunodeficiency virus (HIV) was used for negative control. Northern blotting and a DNA fragmentation assay were carried out as described above.

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