BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 35–40 (1998)
ARTICLE NO. RC989403

An Alternative Form of Poly(ADP-Ribose) Polymerase in *Drosophila melanogaster* and Its Ectopic Expression in Rat-1 Cells

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Received August 28, 1998

We here report an alternatively spliced form of PARP lacking exon 5 of the *Drosophila* PARP gene encoding the auto-modification domain. The alternative form of PARP (PARP II) consists 804 amino acids with a molecular weight of 92.3 kDa. The deduced amino acid sequence of PARP II was completely matched to that of PARP I encoded by a full-length *Drosophila PARP* cDNA, except it lacks the region corresponding to the auto-modification domain. To examine the function of PARP II, stable transformants of Rat-1 cells in which PARP II was ectopically expressed by MMTV-LTR were isolated and characterized. After induction with dexamethasone, PARP II transformants showed slower growth and showed morphological changes with loss of spindled shape compared to cells transformed with the vector or PARP I. The PARP II-transformed cells incorporated propidium iodide after induction; however, Annexin V and TUNEL analysis indicated these changes were not due to apoptosis.

The poly(ADP-ribosyl)ation reaction is one of the post-translational modifications of protein in eukaryotic organisms. It is catalyzed by poly(ADP-ribose) polymerase (PARP). PARP is a nuclear enzyme which polymerizes ADP-ribose residues of NAD, a respiratory coenzyme, to the acceptor proteins in nuclei (1–4). PARP consists of the functional domains from its amino-terminus; the DNA-binding domain, auto-modification domain, and catalytic domain containing an NAD-binding site. PARP is activated by specific binding to the nick of DNA caused by DNA damaging agents (5–7). Nuclear proteins such as DNA polymerase a and b, DNA ligase I and II, histones, Ca^{2+}, Mg^{2+}-dependent endonuclease and PARP itself are poly(ADP-ribosyl)ated (1–3). Although the biological role of this post-translational modification is still controversial, PARP is thought to be involved in chromosome instability and nucleotide excision repair (8–10). In apoptosis, PARP is one of the death substrates for caspase-3 (11–13) as well as nuclear components such as nuclear lamins, DNA replication complex C, and DNA-dependent protein kinase (14–16).

Among different species, two zinc-fingers in the DNA-binding domain and NAD-binding motifs in the catalytic domain were conserved (17). *Drosophila* PARP has 42% homology to human PARP, and the functional structures are well conserved (17,18). Northern blot analysis showed a minor band of 2.6 kb in addition to a major 3.2 kb band of PARP mRNA in *Drosophila* embryos suggesting that two kinds of transcripts from the PARP gene were expressed *D. melanogaster* (18). Here, we describe cDNA cloning of 2.6 kb cDNA for *Drosophila* PARP, determination of the primary structure, and characterization of a stable transformant of Rat-1 cells in which these 2 types of *Drosophila* PARP are ectopically expressed.

MATERIALS AND METHODS

Screening of λgt11 Drosophila cDNA library. The λgt11 *Drosophila* cDNA library (a gift of Dr. T. Hsieh, Duke University Medical Center) was screened by probing of the carboxyl terminal cDNA for *Drosophila* PARP. Replicate filters were prehybridized and then hybridized with the above cDNA that had been labeled [γ-^{32}P]ATP (3000Ci/mmol; 1 Ci = 37 Gbq; Du-Pont, New England Nuclear, UK.) according to the standard procedures (19).

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Abbreviations: PARP, poly(ADP-ribose) polymerase [EC 2.4.2.30]; PARP, poly(ADP-ribose) polymerase gene; D, *Drosophila;* PBS, phosphate-buffered saline; PCR, Polymerase chain reaction; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick end labeling.

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MDIELPYLAE YARTGRATCK GCKSTISKDT LRIAVMVQSA FHDAKVPNWF HKTCFFKNQR  60
PSSVGDIQNI GNLRFADQKE LTDLVENIQE VISAQLGKKR SKAFNLALKD FGIEYAKSSR 120
STCRGCEQKI NKDLVRLRKT VYDTEVGMKY GGQPLWHHLE CFAQLRSELG WFASGEDMPG 180
FQSLADDDQA KVKNAIPPIK SEELPDTKRA KMELSDTNEE GEKKQRLKDQ NDAYFRFRDD 240
IKNKMKKKDI DILLKFNNQQ PVTGDTEKLF DQTADLLTFG AIESCSECNS CQFIVNKSGY 300
ICNGNHSEWT KCNKLLKEPT RSACIVPKEL KALYNFLNTV KEIPSTRIFN NFPPNKSTFS 360
RSLLKTNKNN DVLVRYWIFR SWGRIGTNIG NSKLEEFDTS ESAKRNFKEI YADKTGNEYE 420
QRDNFVKRTG RMYPIEIQYD DDQKLVKHES HFFTSKLEIS VQNLIKLIFD IDSMNKTLME 480
FHIDMDKMPL GKLSAHQIQS AYRVVKEIYN VLECGSNTAK LIDATNRFYT LIPHNFGVQL 540
PTLIETHQQI EDLRQMLDSL AEIEVAYSII KSEDVSDACN PLDNHYAQIK TQLVALDKNS 600
EEFSILSQYV KNTHASTHKS YDLKIVDVFK VSRQGEARRF KPFKKLHNRK LLWHGSRLTN 660
FVGILSHGLR IAPPEAPPTG YMFGKGIYFA DMVSKSANYC CTSQQNSTGL MLLSEVALGD 720
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EYIVYDVAQV NIQYLFRMEF KYSY                                       804
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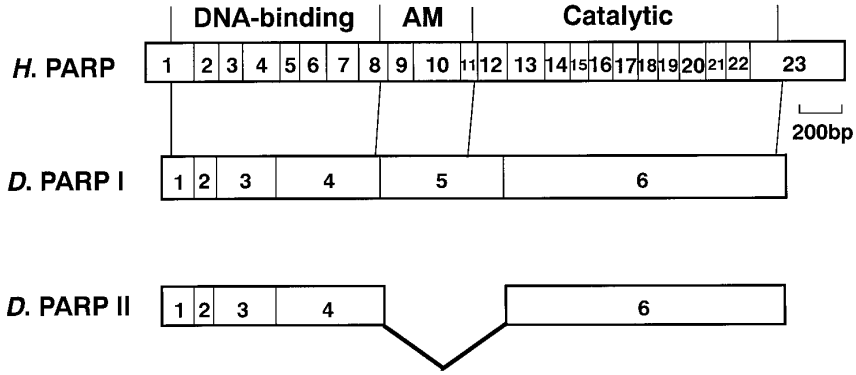

FIG. 1. Amino acid sequence of an alternative form of *Drosophila* PARP and alignment of exon structures. (Top) Deduced amino acid sequence of PARP II. Arrowhead corresponds to the portion of splicing junction of exon 4 and 6. The zinc finger structures and the dinucleotide-binding consensus sequence are underlined. (Bottom) Alignments of exons between human PARP and *Drosophila* PARP I and II. The DNA-binding, auto-modification, and catalytic domains in human, and alignment of these functional domains between human and *Drosophila* PARP, are shown. *Drosophila* PARP II lacks exon 5 corresponding to the auto-modification domain.

DNA sequencing. Restriction fragments of phage clones containing exons were subcloned into pBluescript II KS- (Stratagene, La Jolla, CA) and the deletion plasmids were prepared. Sequencing was performed by the dideoxy chain termination method using a sequencing kit (BRL, Gaithersburg, MD; Applied Biosystems, Foster City, CA).

Plasmid construction. PARP I and II cDNAs were subcloned into multi-cloning site of pMAM-neo (Clonetech, Palo Alt, CA). Expression plasmids were isolated by alkaline-SDS method and purified by QIAGEN-Tip (QIAGEN, Hilden, Germany). HG0, a glucocorticoid receptor expression plasmid, was kindly provided from Dr. P. Chambon (Université Louis Pasteur).

Cell culture. Rat-1 cells (a kind gift of Dr. M. Miura, University of Osaka), a rat fibroblast monolayer cell line, were maintained in Dulbecco Modified Eagle Medium (DMEM) (Nissui, Tokyo) supplemented with L-glutamine (1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) (Sigma, St. Louis, MO), 2.85% sodium bicarbonate solution (GIBCO, Grandisland, NY), and 10% fetal bovine serum (FBS) (Summit, Tokyo).

Transfection of plasmid and isolation of stable transformants. Co-expression of a glucocorticoid receptor in the pMAMneo-transformed cells has shown high expression of exogenous protein induced by dexamethasone (20). Prior to transfection, Rat-1 cells were seeded at 1.5×10^5 cells into 3.5 cm-diameter petri dish (CORNING, Corning, NY). One μg each of pMAM-PARP I or pMAM-PARP II was incubated with 0.25 μg of a HMG-1,2 mixture (WAKO, Kyoto) at 20°C for 1 hr. Then the solution was gently mixed with lipofectamine solution (LIPOFECTAMINE Reagent; GIBCO/BRL) and overlaid onto the monolayer Rat-1 cells. Transformants were selected under 400 μg/ml geneticin (GIBCO) for 2 weeks. A number of positive cells was subcloned by endpoint dilution in a 96-well dish (CORNING). Each of the clones of the pMAM-PARP transfected cells was super-transfected with HG0 and a hygromycin-resistant plasmid (pTK-hygro) (20). Colonies resistant to hygromycin B (GIBCO) (300 μg/ml) were isolated. Rat-1 cells transformed with pMAM-neo and/or HG0 were isolated as controls.

FACS analysis. Apoptotic cells were detected by FITC-Annexin V/propidium iodide (PI) staining (21,22) using Apoptosis Detection Kit (R&D SYSTEMS, Minneapolis, McKinly PI., N.E.) and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method (23). Collected Rat-1 and Jurkat cells were washed twice in PBS containing 0.5% BSA and resuspended in the binding buffer (HEPES-buffered saline supplemented with 0.25 mM $CaCl_2$). FITC-Annexin V and PI were added into the binding buffer at a final concentration of 1 μg/ml and 5 μg/ml, respectively. Flow cytometry was performed by FACSort flow cytometer and Lysis II software (Becton Dickinson, Mountain View, CA). Apoptosis was also analyzed by the TUNEL method using an In Situ Cell Death Detec-

tion Kit (Boehringer Mannheim, Germany) according to the manufacture's protocol. As a control experiment, Jurkat and Rat-1 cells treated with 50 μM etoposide for 6 hr and 24 hr, respectively, were analyzed. The reaction mixture without TdT and Rat-1 cells without etoposide treatment were negative controls. Apoptotic cells were analyzed by FACSort flow cytometer. To examine the sensitivity to etoposide of these transformants, etoposide was added into DMEM medium to a final concentration of 50 μM at 3 hr after induction by dexamethasone, and incubated for 24 hr. The cells were collected in microtubes and washed twice in PBS followed by TUNEL staining.

RESULTS AND DISCUSSION

An alternative form of Drosophila PARP. Two types of PARP cDNA were cloned from a *Drosophila* embryonic cDNA library. Sequencing of these cDNAs and comparison of the nucleotide sequence of these cDNAs to exon-intron structure of the PARP gene revealed that one was a full-length type, PARP I, and the other cDNA, PARP II, was an alternative form which lacked exon 5 possibly produced by alternative splicing of mRNA (Fig. 1). The primary structure of PARP II completely matched to PARP I except for the lack of the auto-modification domain. Calculated molecular weight was 92.3 kDa. PARP II has the DNA-binding domain with zinc-fingers suggesting that DNA-binding ability may be retained. Expression of PARP II during *Drosophila* development was observed in embryos by Northern blotting and RT-PCR using specific primers for PARP II or using primers in exon 3 (forward) and exon 5 (reverse) (data not shown).

Stable transformants of PARP I and PARP II. We constructed GST-fusion PARP I and PARP II proteins. We detected poly(ADP-ribosyl)ation activity in PARP I, while little enzyme activity of PARP II was observed (24). We hypothesized the dominant negative action of PARP II as PARP II retains the DNA-binding domain completely and lacks the self-ADP-ribosylation sites. Actually PARP II was not poly(ADP-ribosyl)ated by PARP I (24). As *Drosophila* cell lines as well as embryos had PARP II mRNA, we introduced PARP II cDNA into rat fibroblast Rat-1 cells, in which an alternative form of mammalian PARP was not detected by RT-PCR.

Four independent clones of the PARP I transformant and 5 independent clones of the PARP II transformant, which were resistant to neomycin and hygromycin were obtained. As a control, 3 clones and 10 clones of Rat-1 cells transfected with pMAM-neo vector and HG0 respectively were also obtained. Fig. 2. shows growth curves of PARP I, II and control transformants with and without induction by dexamethasone. PARP I transformants showed slower growth than the control, but growth of the PARP I transformant was not affected by addition of dexamethasone (Fig. 2A). PARP II transformants also showed slower growth compared to the control. By addition of dexamethasone, the growth of PARP II transformants was significantly inhibited

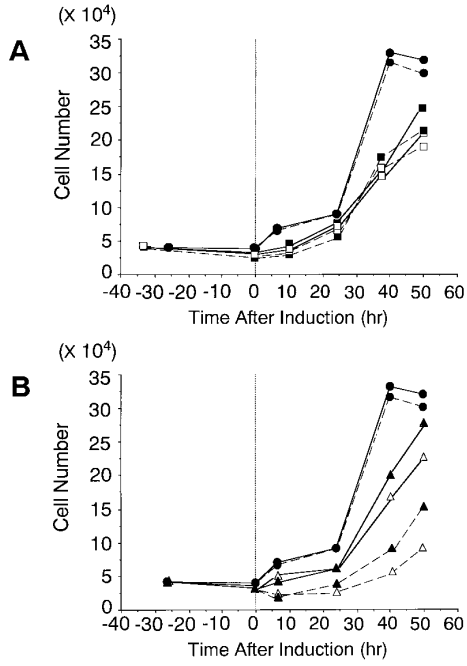

FIG. 2. Growth curves of PARP I and PARP II transformants after induction. (A) PARP I transformants. pMAM-neo (●), pMAM-PARP I transformant clone 1 (■), pMAM-PARP I transformant clone 2 (□). (B) PARP II transformants. pMAM-neo (●), pMAM-PARP II transformant clone 1 (▲), pMAM-PARP II transformant clone 2 (△). Dashed line indicates Rat-1 cells after induction by addition of dexamethasone. Zero time means addition of dexamethasone. After induction, PARP II Rat-1 cells showed slower growth compared to pMAM-neo and PARP I transformants.

(Fig. 2B). The number of cells in each independent clone of PARP II transformants was decreased to 30–50% of the uninduced condition at 40 hr after the induction. Viability checked by trypan blue staining was not affected. The expression of exogenous PARP I and II was confirmed by RT-PCR using specific primers for PARP I and II because a specific antibody against *Drosophila* PARP was not available (data not shown).

Morphological changes of the PARP II transformant. Rat-1 cells are very flat and adherent, and less susceptible to spontaneous transformation than NIH3T3 cells. Evident morphological differences before and after induction were observed in PARP II-Rat-1 cells, while PARP I- and control Rat-1 cells did not show any morphological change. As shown in Fig. 3C, most of PARP II-Rat-1 cells were rounded rather than spindle-shaped and were less adherent. These changes were most evident at 6 hr after induction and continued until 48 hr. No focus formation was observed. The cell adhesion as well as morphology of the cell is controlled by multiple components including fibronectin matrix, actin polymerizaton and signal transducers in the cytoplasm. Although it is speculated that ectopic expression of PARP-II affects the signal transduction pathways involved in the adherence status of fibroblastic

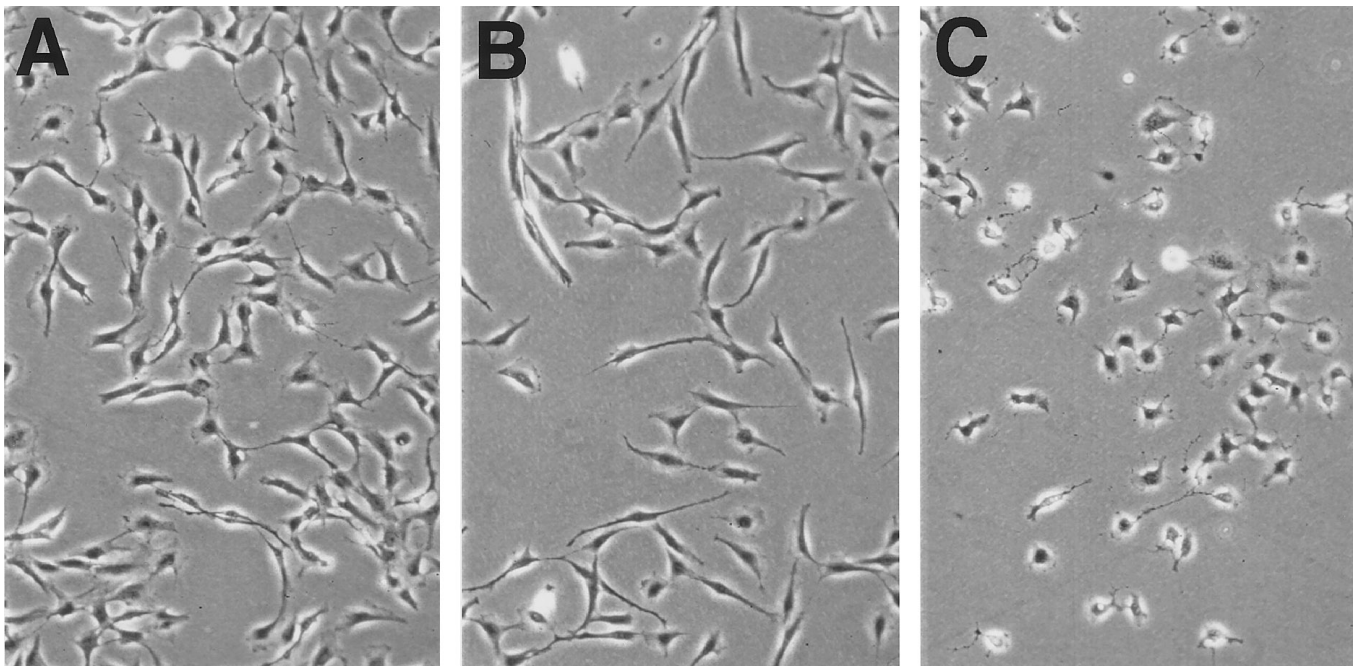

FIG. 3. The morphological features of PARP I and PARP II transformants after induction. The pMAM-neo (A), pMAM-PARP I (B), and pMAM-PARP II (C) transformants were photographed at 6 hr after induction by dexamethasone using phase-contrast optics.

cells, the mechanism of these morphological changes in Rat-1 cells is not clear.

Apoptosis in PARP I and PARP II transformants. The morphology of PARP II-Rat-1 cells was similar to cells treated with the apoptotic agent, etoposide (data not shown). In order to clarify the relationship between morphological changes and growth retardation and apoptosis, the transformants which showed most evident changes were analyzed by flow cytometry using fluorescent-labeled Annexin V and PI. FITC-labeled Annexin V binds to phosphatidylserine when exposed to an apoptotic cell membrane. Apoptotic cells are identified as Annexin V^+/PI^- cells at the early phase and Annexin V^+/PI^+ cells at the late phase of apoptosis. Fig. 4 shows histograms and dot plots from the flow cytometric analysis of PI-stained cells at 6 hr after induction by dexamethasone. At 6 hr after induction, the percentage of PI-positive cells in the PARP II transformants was slightly increased (Fig. 4C). However, the FITC-Annexin V-stained cell population showed no significant difference among transformants (deta not shown). To confirm these results and to analyze the sensitivity of transformants to apoptotic agents, TUNEL analysis was performed. The pMAM-neo-, PARP I-, and PARP II-transformed Rat-1 cells with or without treatment of etoposide were analyzed. Fig. 5 shows histograms and dot plots in the flow cytometric analysis of TUNEL staining. Cells treated by etoposide had stronger fluorescence intensity than untreated cells in Rat-1 cells transfected with control vector, pMAM-neo, indicating an increase of apoptotic cells by etoposide treatment (Fig. 5A, B). Without treatment of etoposide, cells showing strong fluorescence intensity indicative of apoptosis were not detected in PARP II transformants after induction by dexamethasone (Fig. 5D). By treatment of etoposide, no significant change in the population of TUNEL stained cells among control, PARP I and PARP II transformants was observed (Fig. 5B, C, E). These results indicate that morphological changes found in the PARP II expressed Rat-1 cell were not related to apoptosis and that expression of PARP II did not affect apoptosis by etoposide.

It is suggested that PARP II caused growth retardation of Rat-1 cells possibly due to inhibition of cell division rather than cell death. Although slower growth was observed both in PARP I and II transformants without induction by dexamethasone, growth retardation in PARP II after induction was evident. Cell cycle analysis indicated no evident increase of G0/G1 or G2 populations in PARP II-Rat-1 cells at 24 hr after induction (data not shown). However, a time course of cell cycle analysis of PARP II-Rat-1 cells after induction with dexamethasone might be reqired. We examined the presence of an alternative form of PARP corresponding *Drosophila* PARP II in HL-60 cells and human T-cell line, Jurkat cells by RT-PCR using specific primer set which amplify the region of the automodification domain of human PARP, however, this alternative form of PARP mRNA, lacking the automodification domain, was not detected in mammalian cells (data not shown). It could be speculated that

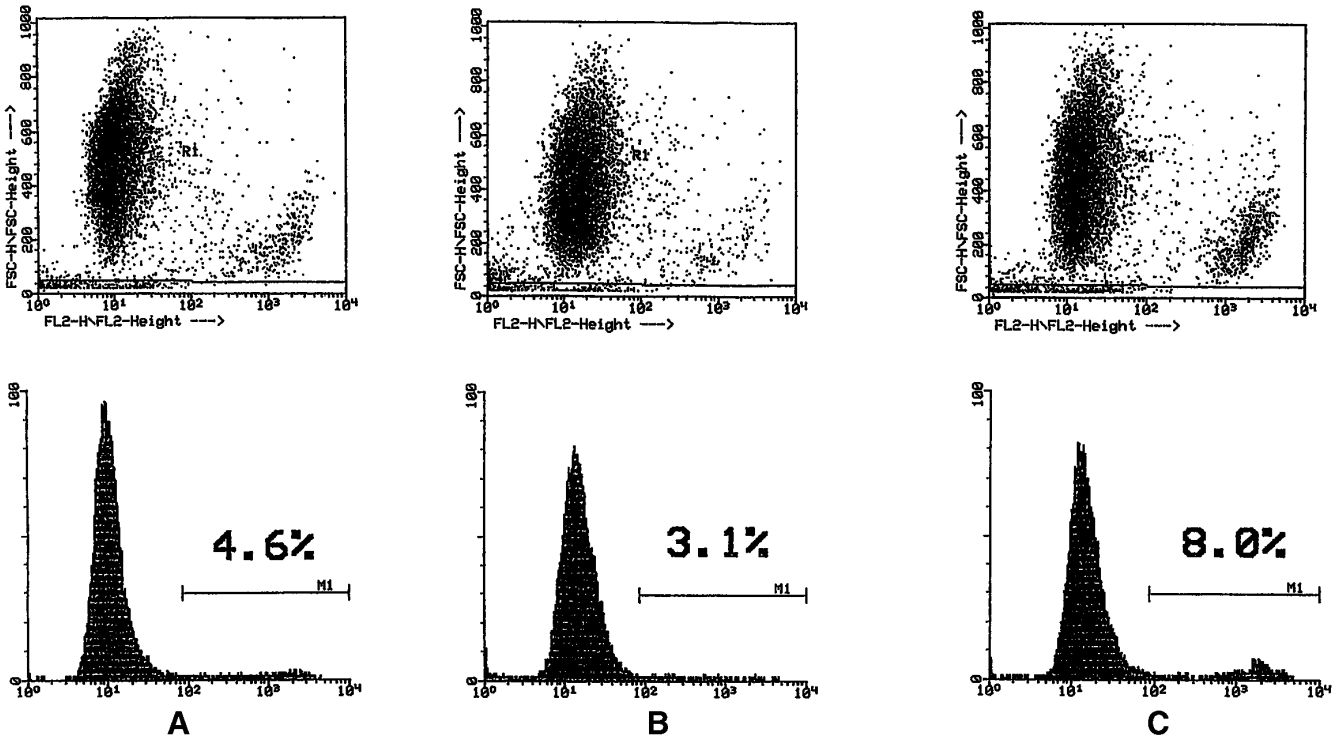

FIG. 4. Flow cytometry analysis of PARP I and PARP II transformants stained with PI. All transformants were stained after treatment with dexamethasone for 6 hr. X-axis in the histogram represents fluorescence intensity and Y-axis represents cell numbers. X-axis of dot plots showed fluorescence intensity, and Y-axis showed forward scatter. (A) pMAM-neo control. (B) pMAM-PARP I. (C) pMAM-PARP II. Percentage of PI-stained cells in PARP II transformant was slightly higher than other transformants.

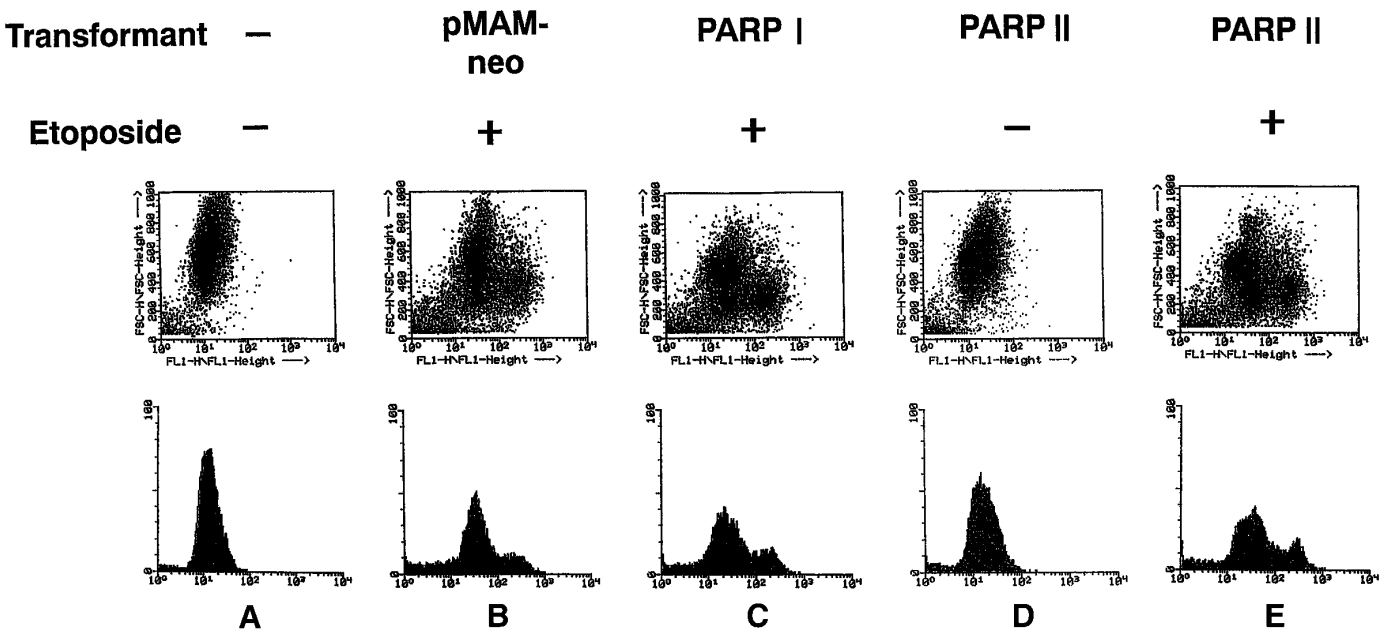

FIG. 5. Apoptosis in PARP I and PARP II-Rat 1 cells stained with TUNEL by Flow cytometry analysis. Treatment with 50 μM of etoposide for 24 hr was performed. Etoposide was added at 3 hr after treatment of dexamethasone. X-axis in the histogram represents fluorescence intensity and Y-axis represents cell numbers. (A) Rat-1 cells without treatment of etoposide as a negative control. (B) pMAM-neo transformant treated with etoposide. (C) pMAM-PARP I transformant treated with etoposide. (D) pMAM-PARP II transformant treated with dexamethasone for 6 hr. (E) pMAM-PARP II transformant treated with etoposide.

morphological changes and an increase in the population of PI-stained cells might be induced by inhibition of the function of native PARP. Functional analysis of PARP II *in vitro*, overexpression of PARP II in *Drosophila* cell lines, and the establishment of PARP II transgenic flies may lead to a better understanding of PARP II function.

ACKNOWLEDGMENTS

We thank Drs. A. Büekle and J.-H. Küpper for the MMTV vector and Dr. P. Chambon for HG0 plasmid. We thank Dr. A. Büekle for helpful comments and discussion. We also thank Dr. S. Yoshida and Ms. Y. Koyama, for help in cell culture, and Dr. Y. Takahama for help in flow cytometry.

REFERENCES

1. Sugimura, T., and Miwa, M. (1983) *Carcinogenesis* **4,** 1503–1506.
2. Althaus, F. R., and Richter, C. (1987) ADP-ribosylation of Proteins: Enzymological and Biological Significance, Springer-Verlag, Berlin.
3. Miwa, M., and Uchida, K. (1992) The Post-translational Modification of Proteins: Roles in Molecular and Cellular Biology (Tuboi, S., Taniguchi, N., and Katsunuma, N., Eds.), pp. 171–182, Scientific Society Press, Tokyo, Japan.
4. de Murcia, G., and Ménissier-de Murcia, J. (1994) *Trends Biochem. Sci.* **19,** 172–176.
5. Ménissier-de Murcia, J., Molinete, M., Gradwohl, G., Simonin, F., and de Murcia, G. (1989) *J. Mol. Biol.* **210,** 229–233.
6. Gradwohl, G., Ménissier-de Murcia, J., Molinete, M., Simonin, F., and de Murcia G. (1989) *Nucleic Acids Res.* **17,** 7112.
7. Ikejima, M., Noguchi, S., Yamashita, R., Ogura, T., Sugimura, T., Gill, D. M., and Miwa, M. (1990) *J. Biol. Chem.* **265,** 21907–21913.
8. Morrison, C., Smith, G. C. M., Stingl, L., Jackson, S. P., Wagner, E. F., and Wang, Z.-Q. (1997) *Nature Genetics* **17,** 479–482.
9. Caldecott, K. W., Aoufouchi, S., Johnson, P., and Shall, S. (1996) *Nucleic Acids Res.* **24,** 4387–4394.
10. Masson, M., Niedergang, C., Schreiber, V., Muller, S., Ménissier-de Murcia, J., and de Murcia, G. (1998) *Mol. Cell Biol.* **18,** 3563–3571.
11. Kaufmann, S. H., Desnoyers, S., Ottaviano, Y., Davidson, N. E., and Poirier, G. G. (1993) *Cancer Res.* **53,** 3976–3985.
12. Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995) *Cell* **81,** 801–809.
13. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T. -T., Yu, V. L., and Miller, D. K. (1995) *Nature* **376,** 37–43.
14. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell* **91,** 479–489.
15. Song, Q., Burrows, S. R., Smith, G., Lees-Miller, S. P., Kumar, S., Chan, D. W., Trapani, J. A., Alnemri, E., Litwack, G., Lu, H., Moss, D. J., Jackson, S., and Lavin, M. F. (1996) *J. Exp. Med.* **184,** 619–626.
16. Song, Q., Lees-Miller, S. P., Kumar, S., Zhang, N., Chan, D. W., Smith, G. C. M., Jackson, S. P., Alnemri, E. S., Litwack, G., Khanna, K. K., and Lavin, M. F. (1996) *EMBO J.* **15,** 3238–3246.
17. Uchida, K., Hanai, S., Ishikawa, K., Ozawa, Y., Uchida, M., Sugimura, T., and Miwa, M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3481–3485.
18. Hanai, S., Uchida, M., Kobayashi, S., Miwa, M., and Uchida, K. (1998) *J. Biol. Chem.* **19,** 11881–11886.
19. Sambrook, J., Fistsch, E. F., and Maniatis, T.(1989) Molecular Cloning, 2nd Ed.
20. Küpper, J.-H., Müller, M., Jacobson, M. K., Tatsumi-Miyajima, J., Coyle, D. L., Jacobson, E. L., and Büekle, A. (1995) *Mol. Cell Biol.* **15,** 3154–3163.
21. Koopman, G., Reutelingsperger, C. P. M., Kuijten, G. A. M., Keehnen, R. M. J., Pals, S. T., and van Oers, M. H. J. (1994) *Blood* **84,** 1415–1420.
22. Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. (1995) *J. Immunol. Methods* **184,** 39–51.
23. Gavrieli, Y., Sherman, Y., and Ben-Sasson S. A. (1992) J. *Cell Biol.* **119,** 493–501.
24. Miwa, M., Hanai, S., Poltronieri, P., Uchida, M., and Uchida, K. (1998) *Mol. Cell Biochem.*, in press.