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Down-regulation of human FEN-1 gene expression during differentiation of promyelocytic leukemia cells

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Abbreviations: DMSO, dimethyl sulfoxide; FEN-1, flap endo/exonuclease; MF-1, maturation factor-1; PCNA, proliferating cell nuclear antigen; PMSF, phenylmethylsulfonyl fluoride; RA, *all-trans* retinoic acid; TPA, 12-*O*-tetradecanoylphorbol- 13-acetate; XPG, Xeroderma Pigmentosum complementation group G

Abstract

Flap endo/exonuclease-1 (FEN-1) recognizes 5'-flap DNA structures that have been proposed to be important intermediates in DNA replication, repair and recombination, and cleaves the double strand-single strand junction of flap substrates. Using an *in vitro* model system, recent studies have shown that FEN-1 is a necessary enzyme for the removal of RNA primers in Okazaki fragment maturation during lagging strand DNA synthesis. In this report, the FEN-1 gene expression was examined during cell cycle and differentiation. Although FEN-1 mRNA and protein could be detected at all stages of the cell cycle, their levels were more elevated in exponentially proliferating cells than in G1 or G2/M-synchronized cells. Moreover, a significant increase of FEN-1 protein was observed when temporarily quiescent fibroblasts were induced to proliferate by serum stimulation. In contrast, the FEN-1 mRNA level showed a sharp decrease in HL-60 cells differentiated by dimethylsulfoxide, *all-trans* retinoic acid or 12-*O*-tetradecanoylphorbol-13-acetate. These results demonstrate that the FEN-1 gene expression is up-regulated during entrance into the mitotic cell cycle and down-regulated in nongrowing cells, as in the case of differentiated promyelocytic leukemia cells.

Keywords: Cell differentiation, FEN-1, Gene expression, HL-60 cells

Introduction

Flap endo/exonuclease-1 (FEN-1), a structure specific nuclease, cleaves the double strand-single strand junction of 5'-branched DNA, called a DNA flap structure, and also possesses 5'→3' exonuclease activity with a specificity for double stranded DNA containing nick or 5'-recessed ends (Harrington and Lieber, 1994a). It has been proposed that DNA flap structures exist *in vivo* as intermediates during DNA replication, DNA repair and homologous DNA recombination (Pont-Kingdon *et al*., 1993; Turchi *et al*., 1994).

The nucleotide sequence of FEN-1 is highly homologous to the RAD2 gene family such as the human Xeroderma Pigmentosum complementation group G (XPG), *S. cerevisiae RAD2*, *S. cerevisiae YKL510* and *S. pombe rad13*, which are required for nucleotide excision repair (Prakash *et al*., 1993; Harrington and Lieber, 1994b; Murray *et al*., 1994). Therefore, it has been hypothesized that FEN-1 participates in the DNA repair mechanism.

Recently, it has been shown that FEN-1 is the same enzyme as maturation factor-1 (MF-1) (Hiraoka *et al*., 1995) which is one of the components necessary for SV40 DNA replication *in vitro* (Waga *et al*., 1994). In addition, FEN-1 shows significant DNA sequence homology (Shen *et al*., 1996) and similar enzyme activity (Turchi and Bambara, 1993) to the 5'→3' exonuclease domain in *E. coli* DNA polymerase I, that also belongs to the structure-specific nucleases family (Lyamichev *et al*., 1993). A bovine analogue of human FEN-1 has been detected in highly purified fractions of DNA polymerase ε, and is required for Okazaki fragments processing during lagging strand DNA synthesis (Bambara *et al*., 1997).

Taken together, it has been proposed that FEN-1 is an eukaryotic counterpart to the 5'→3' exonuclease domain in prokaryotic DNA polymerase, and plays an important role in DNA replication, repair and recombination. However, to date there is no study showing the gene expression of FEN-1 in specific cell cycle stages. In this paper, the gene expression of human FEN-1 was analyzed during the cell cycle and cell cycle exit phase using HL-60 cells in different growth conditions.

Materials and Methods

Materials

Hydroxyurea, nocodazole, dimethylsulfoxide (DMSO), *all-trans* retinoic acid (RA), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), phenylmethylsulfonyl fluoride (PMSF), trypsin inhibitor, leupeptin, antipain and propidium iodide were purchased from Sigma Chem. Co. (St. Louis, MO). DNase-free RNase was obtained

from Boehringer Mannheim (Mannheim, Germany). Ni^{2+}-Sepharose gel was purchased from Invitrogen Co. (Carlsbad, CA). All other chemicals used in the study were of reagent grade.

Cell culture and synchronization

The HL-60 and NIH-3T3 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37˚C in a humidified atmosphere of 5% CO_2.

To synchronize cells at the G1 phase, the HL-60 cells were seeded at a density of 1×10^5/ml and hydroxyurea was added to a final concentration of 1 mM. The cells were then incubated for 12 h at 37˚C in CO_2-incubator. For preparing G2/M-arrested cells, the cells were treated with 0.1 μg/ml nocodazole for 16 h.

Flow cytometry analysis

Exponentially proliferating HL-60 cells and cell cycle-arrested cells were washed with PBS twice, fixed in cold 70% ethanol, and stored at 4˚C. After 2 days, the cells were resuspended in 1 ml of PBS containing DNase-free RNase A (40 u/ml) and propidium iodide (50 μg/ml). Following a 45 min agitated incubation at room temperature, the amount of propidium iodide incorporation was determined using a flow cytometer (Coulter Epics Profile II, Hialeah, FL) (Logan *et al*., 1995)

Induction of differentiation

HL-60 cells (1×10^5/ml) were seeded in a medium containing DMSO (1.3%), RA (1 μM) or TPA (100 nM), and then cultured at 37˚C in a 5% CO_2-incubator for 5 days (TPA) or 7 days (DMSO and RA). Differentiation of cells was evaluated by morphological changes.

Preparation of anti-FEN-1 antibody

For protein overexpression, the DE3 cells (*E. coli* strain BL21) transformed by pET-FCH plasmid containing human FEN-1 cDNA were kindly donated by Dr. Park (Los Alamos Natl. Lab., NM). The recombinant FEN-1 protein was purified by passing the supernatant of the DE3 cell lysates over a Ni^{2+}-Sepharose column under nondenaturing conditions.

The antibody for western blot analysis was prepared by immunizing rabbits with the purified recombinant hFEN-1 protein.

Western blot analysis

The synchronized cells were washed with PBS twice, and then lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 100 μg/ml trypsin inhibitor, 50 μM leupeptin, 100 μM antipain; pH 8.0) . After determining protein concent-ration of each lysate, equal amounts of the samples were loaded on a 12.5% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and analyzed with the anti-hFEN-1 antibody using an enhanced chemiluminescence detection kit (Amersham, Bucking-hamshire, U.K.).

Northern blot analysis

Total cellular RNAs were isolated from HL-60 cells treated with or without various reagents. six μg of the each RNA sample were electrophoresed on 1% agarose-formaldehyde gel and transferred to a nylon membrane. FEN-1 mRNA was analyzed by the DIG chemiluminescence detection method according to the procedure recommended by the manufacturer (Boehringer Mannheim, Mannheim, Germany). The probes incorporated with DIG-11-dUTP were prepared by PCR amplification (606 - 940 bp region of human FEN-1 cDNA).

Results and Discussion

Gene expression of FEN-1 during cell proliferation

It has been shown that FEN-1 is an important component for DNA replication (Bambara *et al.,* 1997), and that several proteins acting in DNA replication, such as DNA polymerase α, proliferating cell nuclear antigen (PCNA), and DNA ligase, are inducible during cell proliferation (Wahl *et al*., 1988; Montecucco *et al.,* 1992; Moore and Wang, 1994). In order to examine whether FEN-1 could also be induced during cell proliferation, FEN-1 gene expression was analyzed in specific cell cycle stages and during cell cycle exit.

For synchronizing in G1 and G2/M phases, HL-60 promyelocytic leukemia cells were incubated with 1 mM of hydroxyurea for 12 h and 0.1 μg/ml of nocodazole for 16 h, respectively. After incubation, the DNA contents of the cells were analyzed using a flow cytometer. As shown in Figure 1A, more than 80% of the hydroxyurea-treated cells arrested at G1 and about 70% of the nocodazol-treated cells arrested at G2/M. When the HL-60 cells were incubated with 0.1 μg/ml of nocodazole for 16 h, some cells died because of the toxicity of this reagent. It is likely that the cell population having a low DNA content shown in Figure 1Ac corresponds to the dead cells. As it is difficult to get cells in the S phase only, exponentially growing HL-60 cells (log-phase cells) were used instead of cells synchronized in the S phase (cells synthesizing DNA).

Total RNAs were isolated from G1 and G2/M-arrested HL-60 cells as well as exponentially growing cells. The FEN-1 mRNA level was checked by northern blot analysis using a human FEN-1 specific probe labeled with DIG. Not only log-phase HL-60 cells, but also G1 or G2/M-

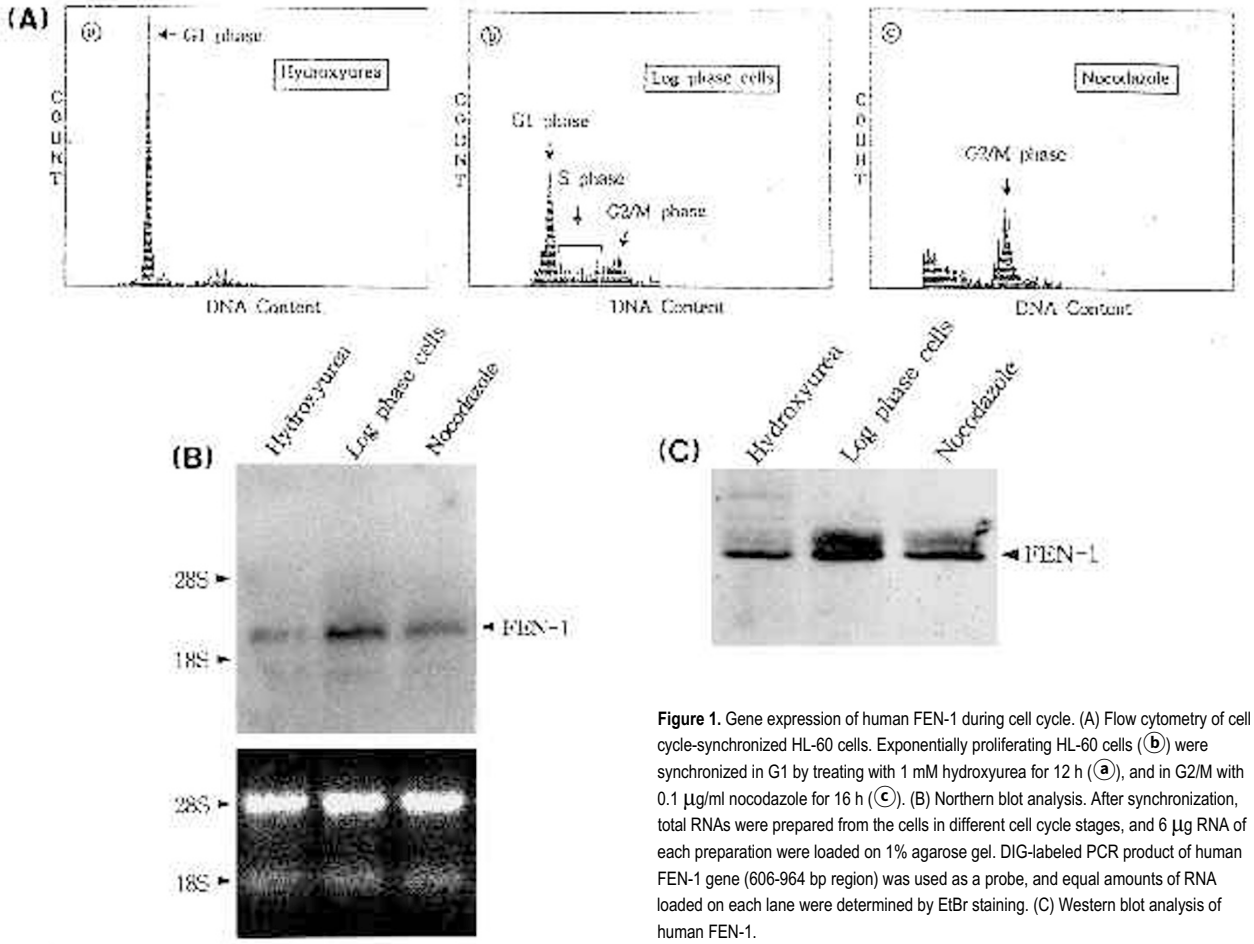

Figure 1. Gene expression of human FEN-1 during cell cycle. (A) Flow cytometry of cell cycle-synchronized HL-60 cells. Exponentially proliferating HL-60 cells (ⓑ) were synchronized in G1 by treating with 1 mM hydroxyurea for 12 h (ⓐ), and in G2/M with 0.1 μg/ml nocodazole for 16 h (ⓒ). (B) Northern blot analysis. After synchronization, total RNAs were prepared from the cells in different cell cycle stages, and 6 μg RNA of each preparation were loaded on 1% agarose gel. DIG-labeled PCR product of human FEN-1 gene (606-964 bp region) was used as a probe, and equal amounts of RNA loaded on each lane were determined by EtBr staining. (C) Western blot analysis of human FEN-1.

arrested cells exhibited FEN-1 gene expression, but the mRNA level was higher in exponentially growing cells than in growth-arrested cells (Figure 1B). The protein levels in growing and temporarily growth-arrested cells showed similar patterns to the mRNA levels (Figure 1C).

After NIH-3T3 mouse fibroblast cells were growth-arrested at the G1 stage by serum starvation, the cells reentered the mitotic cell cycle following serum stimulation. As shown in Figure 2, a sharp increase of the FEN-1 protein resulted from the induction of entry into the S phase of G1-arrested NIH-3T3 cell.

These results show that FEN-1 is up-regulated during cell proliferation, and shows a similar pattern to other DNA replication proteins, such as DNA polymerase α and DNA ligase, which are also inducible in cell proliferation (Wahl *et al*., 1988; Montecucco *et al.,* 1992; Moore and Wang, 1994).

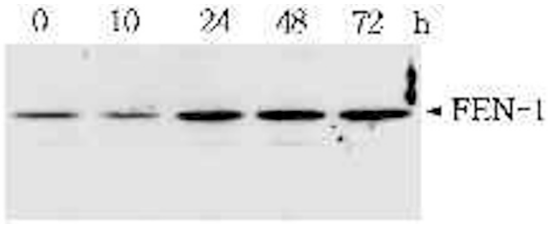

Figure 2. Up-regulation of FEN-1 protein synthesis in mitotic cells. A confluent monolayer of NIH3T3 cells were starved for 2 days in a serum-free medium. Then the cells were stimulated for the indicated times in the presence of 10% serum in order to reenter the mitotic cell cycle. The FEN-1 protein levels were analyzed by immunoblotting.

Induction of differentiation of HL-60 cells

It was discussed as to whether FEN-1 gene expression could be down-regulated in non-growing cells which had exited the cell cycle permanently. It has been well established that HL-60 promyelocytic leukemia cells could be induced through terminal differentiation into granulocytes or macrophages by DMSO, RA or TPA, respectively, and so HL-60 cells were incubated with 1.3% DMSO or 1 μM RA for 7 days, or with 100 nM TPA for 5 days in order to prepare nongrowing human leukemia cells. After incubation, cell differentiation was evaluated by observing morphological changes of the cells. Nuclei of DMSO-

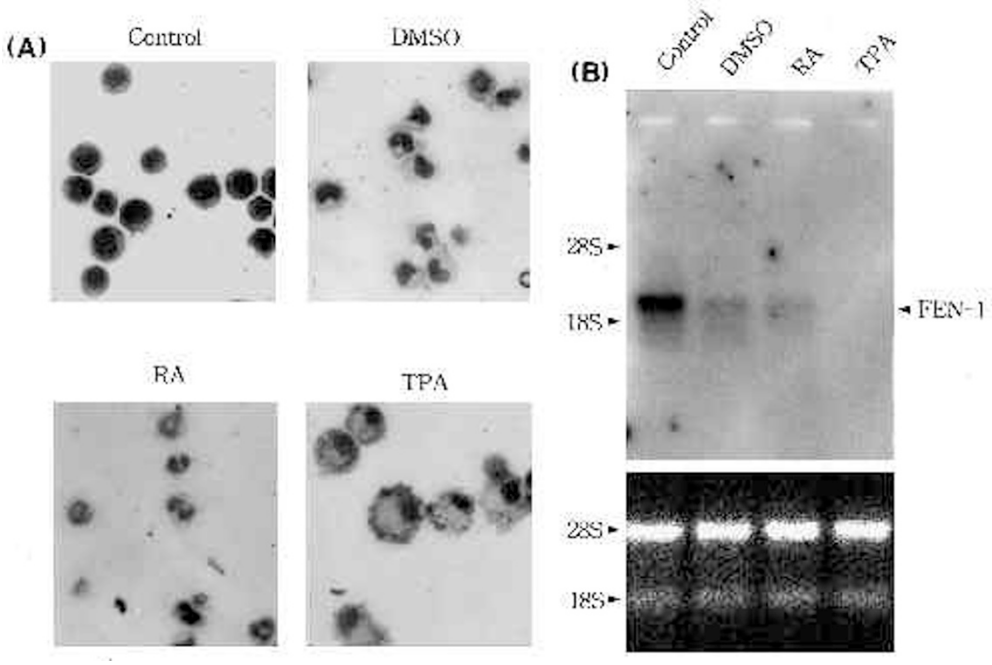

Figure 3. Down-regulation of human FEN-1 gene expression during cell differentiation. HL-60 cells (1×10^5/ml) were treated with 1.3% DMSO or 1 μM RA for 7 days, or with 100 nM TPA for 5 days. (A) To evaluate cell differentiation, the cells incubated with various agents were stained with Giemsa solution and photographed (×400). (B) Northern blot analysis was performed for detection of FEN-1 mRNA levels in the differentiated HL-60 cells.

and RA-treated cells were similar to that of granulocytes showing bending and segregated nuclei, while TPA-treated cells had a decreased nucleus/cytosol ratio contrast compared to untreated control cells in Giemsa staining (Figure 3A). The TPA-treated cells also adhered to the bottom of the culture dish. These results indicate that HL-60 cells were well differentiated into granulocytes or macrophages by DMSO, RA, or TPA.

Down-regulation of FEN-1 gene expression in differentiated HL-60 cells

Human FEN-1 gene expression in differentiated HL-60 cells was analyzed by the northern blot method. As shown in Figure 3B, HL-60 cells differentiated into granulocytes by DMSO and RA showed a remarkable decline in FEN-1 gene expression, 30% and 20% respectively, to the control mRNA level. Specifically, FEN-1 mRNA was not detectable in HL-60 cells treated with TPA for 5 days. These results demonstrate that human FEN-1 gene expression is substantially down-regulated at the mRNA level during cell differentiation, suggesting that human FEN-1 parti-cipates in cell proliferation.

In this study, to identify FEN-1 as a DNA replication protein, human FEN-1 gene expression was examined in actively growing and nongrowing human promyelocytic leukemia cells. Data indicates that human FEN-1 gene is up-regulated during entry into the S phase of G1-arrested cells, and down-regulated in nongrowing cells.

Also, recent studies have demonstrated that FEN-1 is an important enzyme for DNA replication. *i.e.,* i) Mutant cells lacking in yeast FEN-1 gene showed deficient cell growth (Johnson *et al*., 1995). ii) Yeast FEN-1 interacted with a replicative helicase (Budd and Campbell, 1997) as well as PCNA (Li *et al.*, 1995) which is the accessory factor for DNA polymerases δ and ε. iii) Calf FEN-1 homo-logy was copurified with calf DNA polymerase ε (Bambara *et al.,* 1997). iv) Bovine FEN-1 acted in the removal of RNA primer during Okazaki fragment maturation (Bambara *et al.,* 1997).

Together with data from these experiments and other researchers' data, it seems likely that FEN-1 gene expression is induced during cell proliferation to play a role in DNA replication. These facts suggest that FEN-1 may be applied as a useful new tumor marker, the work is now in progress.

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