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A *hVti1* Homologue: Its Expression Depends on Population Doubling Levels in both Normal and SV40-Transformed Human Fibroblasts

Hu-Chun Li, Hidetoshi Tahara, Naohiro Tsuyama, and Toshinori Ide[1]
Department of Cellular and Molecular Biology, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima City, Hiroshima 734, Japan

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A cDNA clone was isolated by differential colony hybridization from a cDNA library prepared from life-extended SV40-transformed human fibroblasts. The clone, tentatively named N-10, was 1272 bp in length coding for 232 amino acids. Northern analysis revealed that the expression level of N-10 was increased in normal senescent and life-extended SV40-transformed fibroblasts than in their young counterparts but was not enhanced by growth arrest. The protein fused to GFP (green fluorescent protein) localized in cytoplasmic granule. Enforced expression of N-10 resulted in premature senescence in young fibroblasts. The deduced amino acid sequence of N-10 was identical to the recently reported *hVti1* gene except in one amino acid: Asp^{24}(GAC) was ours and Asn^{24} (AAC) was reported. Additional base differences were found, so we referred to our sequence as the *hVti1* homologue. As *hVti1* protein was suggested to be involved in the vesicle transport process, the homologue may be concerned with increased secretion of extracelluar matrix and various cytokines associated with cellular senescence. © 1998 Academic Press

Normal human diploid cells in culture have a finite proliferative lifespan and cease proliferation after a limited number of population doublings(PD) (1). Stable nondividing state at the end of their lifespan, termed proliferative senescence, is characterized by altered gene expression (2, 3, 4, 5). Several lines of evidence indicate that senescent cells express dominant inhibitor(s) of cell proliferation which probably controls the process of cellular senescence (6, 7, 8, 9). Expression of $p21^{sdi1}$ and $p16^{ink4B}$, which inhibit the kinase activity of G1 cyclin/Cdk complexes, increases with increasing population doubling levels (PDL) and results in inhibition of Rb phosphorylation and DNA synthesis (10, 11, 12). Cells transformed with SV40 T-antigen gene extends their proliferative lifespan, T-antigen dependently, by about 10-30 PDL as compared with normal counterpart (13, 14). Upon inactivation of T-antigen, life-extended transformed cells immediately stop proliferation and senesce. Expression of $p21^{sdi1}$ increases with increasing PDL in T-antigen transformed cells as well as in normal cells, though the expression levels are always lower in transformed cells (15). In addition to increased expression of such growth-inhibitory genes with cellular senescence, increase in production and excretion was also reported in such extracellular matrix molecules as collagen and fibronectin (16, 17, 18) and such intercellular signal molecules as endothelin in endothelial cells (17) and interleukin-6 in fibroblasts (19), which, if it occurred *in vivo*, would result in functional disorder of human body with aging. We screened cDNA library aiming at isolating cDNAs which were expressed at higher level in senescent normal and life-extended transformed cells than in their younger counterparts. An isolated cDNA clone, which was found to contain the coding sequence of recently isolated human Vti1 gene (*hVti1*) that involved in Golgi to prevacuolar transport and in traffic to the cis-Golgi (20), was expressed at higher level in senescent normal and life-extended transformed cells than in their younger counterparts.

MATERIALS AND METHODS

Cells and culture. Human normal fetal fibroblasts, TIG-3 (21), senesced at around 80 PDL. SV*ts*9-4, a clone of TIG-3 transformed with pMT-1ODtsA plasmid which carried origin-defective and temperature-sensitive SV40 T-antigen gene, proliferated up to about 100-110 PDL at 34°C and then died (mortal). SV*ts*8, an immortal cell line established from pMT-1OD*ts*A transfected TIG-3, continued proliferation indefinitely (over 1000 PD) at 34°C (14). After temperature shift up to 38.5°C, SV*ts*9-4 at extended-lifespan (over 80 PD) and SV*ts*8 immediately stop proliferation with senescent morphology and senescence-associated β-galactosidase expression (22). Cells

[1] Corresponding author. Fax: 81-82-257-5294. E-mail: tide@pharm.hiroshima-u.ac.jp.

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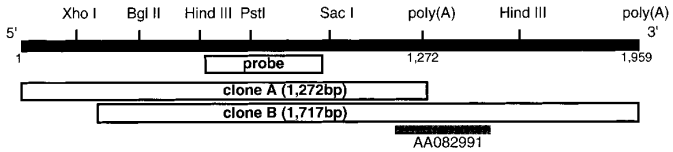

FIG. 1. Structure of isolated N-10 (*hVti1* homologue) clones. Clone A is considered to be a full length N-10 cDNA corresponding to the major product, 1.3 kb mRNA. Clone B is considered to be an alternatively spliced product. AA082991 is an expressed sequence tag registered in GenBank.

were cultured in Dulbecco' s modified Eagle' s medium with 10% fetal bovine serum as previously reported (13).

Construction and screening of cDNA library. cDNA library was prepared from mRNA of life-extended SV*ts*9-4 as described previously (15). Screening was done by differential colony hybridization using 32P-labeled cDNA probes prepared by reverse transcription of mRNA from life-extended SV*ts*9-4 cultured at 34°C (proliferating phenotype) or at 38.5°C (senescent phenotype). Full length cDNA was screened from cDNA phage library prepared from SV*ts*8 mRNA and subcloned into pBK-CMV vetor (Strategene: La Jolla, USA) by *in vivo* excision of phagemid. Sequencing was done by dideoxy method.

Northern hybridization. Ten μg total RNA prepared by acid guanidium isothiocyanate/phenol chroloform extraction was run on 1.1% agarose gel, transferred onto Hybond-N membrane, and hybridized with 32P-labeled cDNA probes as described previously (15).

Transfection. Sense and antisense cDNA was subcloned into the pBK-CMV and transfected into young TIG-3 (14 PDL) by electroporation. For colony forming assay, 1×10^5 transfected cells were plated, cultured in a selection medium (500μg/ml G418), and fixed after 2 weeks. For isolation of transfected clones, transfected cells were cultured in selection medium for 2 weeks and cylinder cloned. Cloned cells were propagated and passaged until they stopped proliferation. cDNA was connected in-frame to the C-terminus of enhanced green fluorescent protein (EGFP: pEGFP-C1; Clontech : Palo Alto, USA) and transfected into HT1080 cells by electroporation.

RESULTS AND DISCUSSION

Isolation of N-10 cDNA clone. Six cDNA clones which were expressed higher in life-extended SV*ts*SV9-4 cells cultured at 38.5°C than in those at 34°C were isolated through three successive differential colony hybridization. By partial sequencing and homology search, one of six was found to be a fragment of α2-procollagen type VI cDNA and the others were unreported sequences. All cDNA clones of unknown sequences except for one clone, N-10, gave no clear signals in northern hybridization of total RNA (10μg/lane) from life-extended SV*ts*9-4 cells. cDNA probe of N-10 hybridized to 1.3 kb mRNA. To isolate full length cDNA of N-10, several clones were isolated from cDNA phage library prepared from SV*ts*8 and were subcloned into plasmid. Two kinds of cDNA clones (clone A and clone B) were likely to be products from differential poly (A) site usage. Other clones were 5′ trancated ones of clone A. Clone B was the longest one (1,718 bp) but appeared to have a 5′ truncation (Fig. 1). A 266 bp stretch beginning at 1,135th base of clone A and ending at 1,400th base of clone B (numbering of clone B began with 242 bp corresponding to clone A) was identical to the reported expressed sequence tag (Gene Bank #AA082991). Since northern analysis revealed a single band of about 1.3 kb mRNA clone A was assumed to be a nearly full length cDNA of N-10 mRNA expressing in this cell line. One open reading frame beginning at 341st bp encoded 232 amino acid sequence and all others did shorter than 50 amino acids. There existed a termination codon (at 284th bp) and Kozak's consensus sequence around this initiation codon. Deduced peptide had no N-terminal hydrophobic signal sequence, low proline content

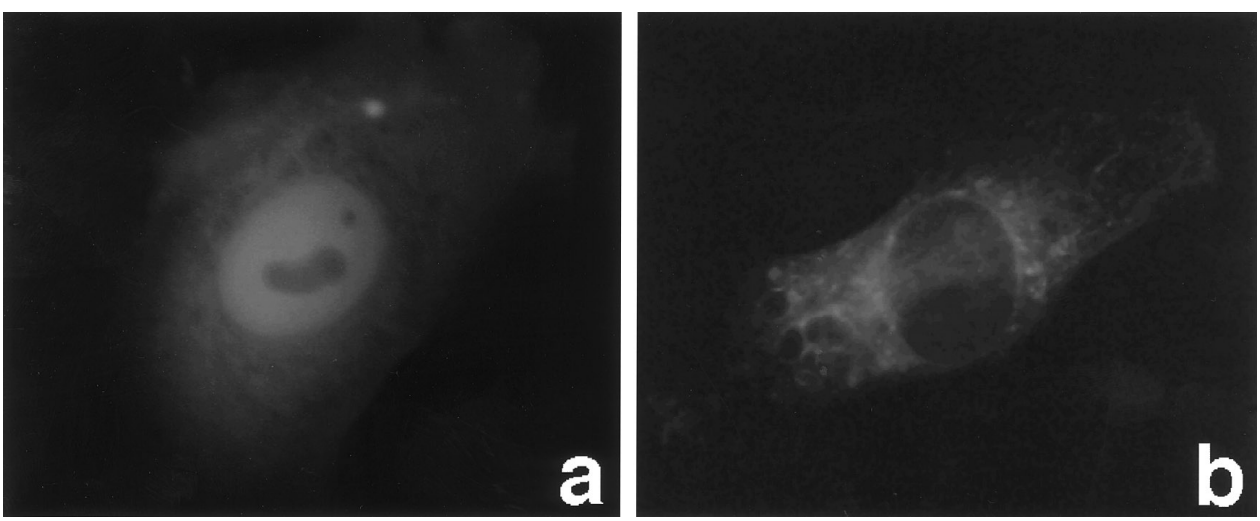

FIG. 2. Cytoplasmic localization of *hVti1* homologue. EGFP-fused *hVti1* localized in cytoplasmic granules (**b**) whereas EGFP alone distributed in cytoplasm and nuclei (fluorescent intensity was higher in nuclei probably because of thickness) (**a**) in transiently transfected HT1080 cells.

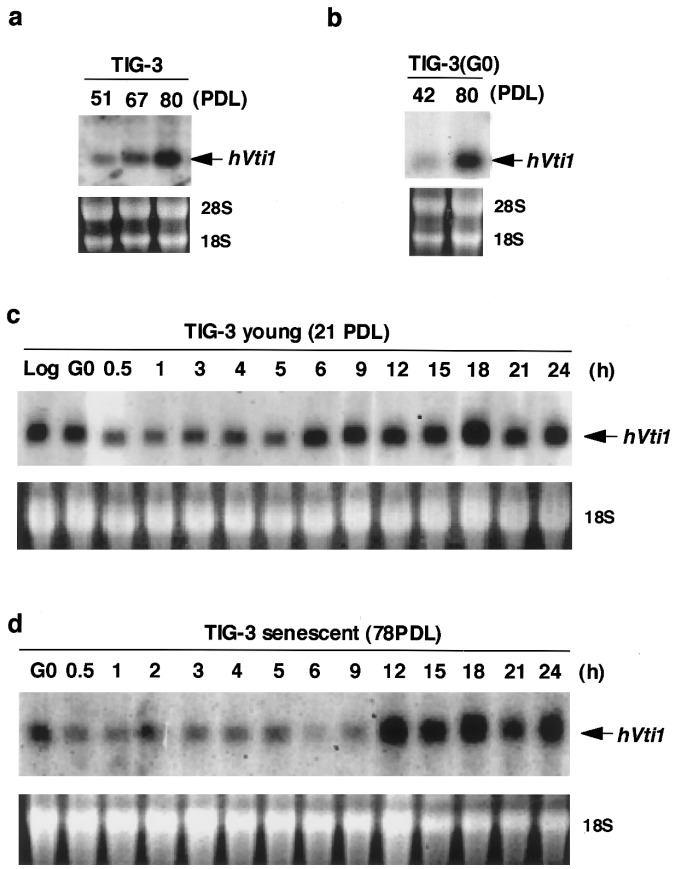

FIG. 3. Expression level of *hVti1* homologue in human normal fibroblasts, TIG-3, by Northern blot. Ten mg total RNA prepared by acid guanidium isothiocyanate/phenol chroloform extraction was run on 1.1% agarose gel, transferred onto Hybond-N membrane and hybridized with 32P-labeled cDNA probes, **a.** Expression of *hVti1* homologue in different PDL. Cell at 51 and 67 PDL were growing and those at 80 PDL were growth-arrested by senescence. Cells were harvested 1 day after medium change. **b.** Cells were growth-arrested by culturing them in serum-free medium for 3 days. **c and d.** Expression of *hVti1* homologue in young (**c**) and senescent (**d**) TIG-3. The cells were growth-arrested in serum-free medium for 3 days (G0) and then stimulated to proliferate by changing medium with that containing 10% serum. At various time (numbers at the top of the figure) after growth stimulation. Since the expression level of *hVti1* homologue was low in young cells, longer exposure period was required for autoradiogram in Fig 3c. Log; continuously proliferating young cells in medium containing 10% serum. Ethidium bromide-stained gel pattern was also presented to monitor the amount of RNA loaded in each lane.

(5/232), and no cystein residue. It had a cluster of basic amino acids, two sets of leucine-zipper structure, and hydrophobic C-terminal tail. During preparation of this manuscript, *hVti1* was reported (20) which encoded polypeptide identical to N-10 except for one amino acid. Amino acid at position 24 of N-10 was aspartic acid (code: GAC), which was identical to the reported amino acid of mouse *Vti1* (*mVti1*), whereas asparagine (code: AAC) in reported *hVti1* sequence (accession no. AF035824). An additional base substitution, without amino acid change, in coding region was found in the codon for glutamic acid at position 30 where GAG by our hands and GAA reported. We amplified DNA fragments including these two base-substitutions by RT-PCR using a fresh polyA-RNA preparation from TIG-3, sequenced and confirmed our sequence, i.e., both normal TIG-3 strain and its derivative cell line (SV*ts*9-4) expressed identical sequences which had minor difference from the reported *hVti1* sequence. We also found 8 other base differences between ours and reported ones in noncoding region. Our sequence was registered in GenBank (AF060902).

Intracellular localization of N-10. To see possible functional difference between N-10 and *hVti1* because of amino acid variation, we confirmed subcellular localization of N-10 protein. EGFP-fused N-10 localized in cytoplasmic granules, whereas EGFP itself distributed in cytoplasm and nuclei (fluorescent intensity was higher in nuclei probably because of thickness) in transiently transfected HT1080 cells (Fig. 2). Although it was uncertain yet whether these sequence differences between N-10 and *hVti1* are polymorphism, we referred to N-10 as *hVti1* homologue hereafter.

Expression of hVti1 homologue (N-10). Expression level of hVti1 homologue increased with increasing PDL (Fig. 3a). Since senescent (80 PDL) cells were growth arrested and younger (51 PDL) cells were growing, an increase in the expression level in senescent cells could be due to growth arrest rather than senescence. When both young (42 PDL) and senescent (80 PDL) TIG-3 cells were growth-arrested in G0 phase by serum starvation for 3 days at confluent stage, the expression level of *hVti1* homologue was also higher in senescent cells (Fig. 3b). When young (21 PDL) TIG-3 cells were growth-arrested by serum-starvation for 3days, the expression level of *hVti1* homologue did not increase as compared with that in log phase cells (compare Log with G0 in Fig. 3c). When G0-arrested cells were growth-stimulated by changing culture medium

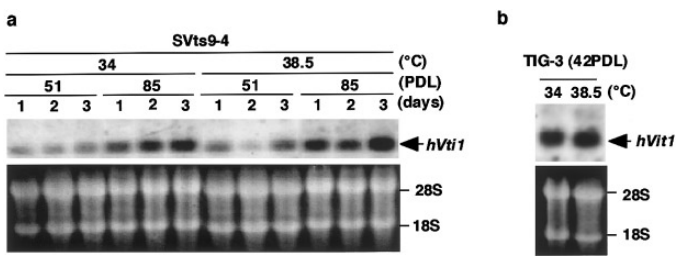

FIG. 4. Expression level of *hVti1* homologue in SV40 T-antigen transformed TIG-3, clone SV*ts*9-4, by Northern blot. **a.** Young (51 PDL) and lifespan extended (85 PDL) SVts9-4, cultured at 34°C were continuously proliferating. The medium was changed at day 1 and the culture temperature was shifted to 38.5°C or maintained at 34°C. **b.** Expression level of *hVti1* homologue in normal TIG-3 cells cultured at 34°C or at 38.5°C for 2 days.

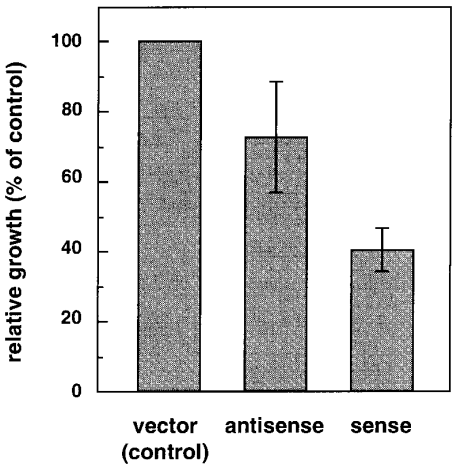

FIG. 5. Inhibition of colony formation in TIG-3 cells transfected with *hVti1* homologue. Sense and antisense *Vti1* cDNA was subcloned into the pBK-CMV and transfected into young TIG-3 (14 PDL) by electroporation. For colony forming assay, 1×10^5 transfected cells were plated in 10 dishes (100mm), cultured in a selection medium (500μg/ml G418), and fixed after 2 weeks. Results were expressed as ratio to vector transfected control (mean ± standard error) from three separated experiments.

with that containing 10% serum (serum-stimulation), the expression of *hVti1* homologue reduced immediately and then transiently increased with a peak at 18 h after stimulation (Fig. 3c). Transient reduction of *hVti1* homologue expression level was also observed in senescent (78 PDL) TIG-3 cells after serum stimulation, though the time course of recovery delayed as compared with that in young cells (Fig. 3d).

In a SV40-transformed SVts9-4, the expression level of *hVti1* homologue also increased with PDLs (compare 34°C of 51 PDL with that of 85 PDL in Fig. 4a). SV*ts*9-4 cells continued to proliferate at 34°C up to 100-110 PDL and so they still grew actively at 85 PDL. After shift up of the culture temperature, lifespan extended (over 80 PDL) SV*ts*9-4 immediately ceased proliferation whereas younger counterparts (before 80 PDL) continued proliferation (15). After the shift up of the temperature, an increase in *hVti1* homologue expression was faint, if not at all, in SV*ts*9-4 (compare 34°C and 38.5°C in Fig. 4a) and in normal TIG-3 (Fig. 4b), whereas the growth was reduced in SV*ts*9-4 cells but did not change at all in normal TIG-3. *hVti1* homologue was isolated in this study as a cDNA which expressed higher in SV*ts*9-4 cultured at 38.5°C than that at 34°C, but the differences in expression level between two temperatures were small. These results indicated that the increase in *hVti1* homologue expression occurred with increased PDLs in both normal and SV40-transformed TIG-3 cells, and neither simply due to growth arrest nor to temperature shift.

Growth suppression by hVti1 homologue transfection. cDNA of *hVti1* homologue connected to the downstream of CMV promoter was transfected into young TIG-3 (21-30 PDL) to examine growth suppressive activity of this gene. The colony number of cells transfected with sense orientation of *hVti1* homologue cDNA was about a half of that of antisense or vector transfected cells (Fig. 5). While the reason was uncertain yet why antisense transfected group gave reduced colony number than vector control group, some expression level of *hVti1* homologue might be necessary for cell proliferation. Fourteen colonies were cloned from sense *hVti1* homologue-transfected TIG-3 cells. Most of these clones ceased proliferation before 30 PDL after transfection with senescent morphology (flat and large). Only 4 clones could be propagated and passed over 30 PDL, *i.e.*, they ceased proliferation at 37 (clone 8), 43 (clone 9), 49 (clone 12), and 55 (clone 11) PDL. The expression levels of *hVti1* homologue was moderate in these clones. Clones which expressed *hVti1* homologue at high level might stop proliferation at earlier PDL including at colony level. Isolated clones from antisense-*hVti1*-homologue- or vector-transfected cultures continued proliferation over 60 PDL after transfection.

Possible role of hVti1 in cellular senecsence. Expression pattern of *hVti1* homologue met criteria on senssescence-gene, i.e., 1) its expression was PDL-dependent in both normal and SV40 T-antigen transformed cells, 2) its expression was independent on growth-arrest of young cells, and 3) its introduction into young fibroblasts resulted in reduced colony formation and premature senescence. Although an apparent growth suppressive effect of transfected *hVti1* homologue may not be intrinsic but artificial due to unregulated overexpression of transfected cDNA in addition to the expression of endogenous gene, other characteristics were consistent with what expected as a senescence gene which expressed PDL-dependently and independently upon growth-arrest. *hVti1* is reported to involve in transport from Golgi to prevacuolar and in traffic to the cis-Golgi (20). When cells senesced, increased production and secretion, in addition to increased expression of growth-arrest related genes, were also observed in such extracellular matrix molecules (23) as collagen and fibronectin and such intercellular signal molecules as endothelin in endothelial cells (17) and interleukin-6 in fibroblasts (19). An increased expression of *hVti1* homologue would facilitate an increased excretion of intercellular signal molecules in senescent cells, and, if it occurred *in vivo*, it would result in an abnormal amount of bioactive materials and in functional disorder of cells and tissues with human aging.

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