

Cloning of cDNA with Possible Transcription Factor Activity at the G₁-S Phase Transition in Human Fibroblast Cell Lines

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Normal human fibroblasts have a finite proliferative capacity *in vitro*. Thus, immortalization of human cells is associated with cellular aging. We have established an immortalization-sensitive cell line from fibroblasts of Wilms' tumor patients which have a partial deletion of chromosome 11p. This cell line was easily immortalized by introducing SV40T. By differential hybridization using both SV40T-introduced crisis cells and young cells, we cloned a gene that was highly expressed in 11p- cells at the time of the crisis and named this gene C-1. Nucleotide sequence analysis of C-1 revealed that it contains a helix-loop-helix domain, indicating that it may be a transcription factor. Expression of the C-1 gene was transiently induced early in the G₀-to-S phase transition in two normal human (OUMS-24 and HSF-412) and a non-tumorigenic immortal human (OUMS-24F) fibroblast cell lines, while the other immortal SUSM-1 cells highly expressed the C-1 gene in the middle G₁ phase. These results suggest that the C-1 gene product may function as a transcription factor related to the cell cycle.

Key words: human cells, SV40T, gene cloning, transcription factor

In *vitro* cell transformation is a valuable model for studying the mechanisms of multistep carcinogenesis. The human cell system is useful for studying the various stages of *in vitro* transformation because events in the transformation process from normal to neoplastic phenotypes are clearly defined by the successive stages of aging, immortalization, and neoplastic transformation. For normal human cells to be neoplas-

tically transformed, they must first be immortalized. However, normal human cells have a finite capacity to replicate and eventually enter a state of irreversible growth arrest (1-3). This phenomenon has been termed "cellular senescence" or "replicative senescence".

Cellular senescence is thought to be a genetically programmed process rather than the result of a random accumulation of damage. In normal human cells, in contrast with normal rodent cells, cellular senescence is particularly stringent and spontaneous immortalization is an extraordinarily rare event. The introduction of SV40T allows some normal human cells to escape cellular senescence, but the frequency of immortalization is very low. Immortalization requires the functional disruption of two mortality stages, M1 and M2 (4). Introduction of SV40T suppresses the M1 mechanism by inactivating tumor suppressor gene products, p53 and Rb protein, thus allowing the cells to have an extended life span until the antiproliferative M2 mechanism is induced and/or activated which causes crisis. Only a few cells escape the M2 crisis and become immortalized by an additional rare aberration of the M2 mechanism, which is as yet unknown.

We have established an immortalization-sensitive cell line from the fibroblasts of a patient with Wilms' tumor, which has a partial deletion of chromosome 11p (5). This cell line was relatively easily immortalized by introducing SV40T. To understand the molecular events during the M2 crisis, we tried to identify which human genes are preferentially expressed in SV40T-introduced 11p- cells at the time of the crisis.

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Materials and Methods

Cells and cultures. The characterization of an immortalization-sensitive cell line derived from the fibroblasts of Wilms' tumor patients is described elsewhere (5). Normal human fibroblasts (HSF-412 and OUMS-24) were immortalized by repeated treatment with 4-nitroquinoline 1-oxide (4-NQO), and the immortalized cell lines were named SUSM-1 and OUMS-24F, respectively (6, 7). The culture medium used was Eagle's minimum essential medium (MEM) (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air, and when they reached confluence in 100-mm dishes, subculturing was performed at a split ratio of 1:2 or 1:4 using 0.2% trypsin (1:250, Difco Laboratories, Detroit, MI, USA) plus 0.02% EDTA solution in Ca²⁺-free and Mg²⁺ free phosphate-buffered saline (PBS). For this study, young normal cells (HSF-412 and OUMS-24) were used at passage levels 38 and 32, and the immortalized cells (SUSM-1 and OUMS-24F) at passage levels 607 and 142, respectively. OUMS-24 and OUMS-24F cells were synchronized at the G₀ phase by incubating them for 3 days in MEM containing 0.2% FBS and then stimulating them to re-enter the cell cycle with 10% FBS-MEM.

Gene cloning. To identify the human genes that show increased expression at the time of cell crisis, a cDNA library was constructed from SV40T-introduced 11p-crisis cells, using the pCDM8 expression vector (8).

A clone that highly expressed cDNA during cell crisis was obtained by differential hybridization using cDNA probes synthesized from mRNA of either SV40T-introduced crisis or precrisis cells. Its cDNA sequence was compared with sequence data in both the GenBank library and the EMBL bank using the BLAST program.

Probes and plasmids. A rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was kindly provided by Dr. Kenji Shimizu (Department of Molecular Genetics, Institute of Molecular and Cellular Biology, Okayama University Medical School).

Northern blot analysis and quantitation of transcripts. Total RNA was extracted by the acidic guanidinium-phenol-chloroform method (9) from synchronized cells at the indicated times. RNA samples (15 µg/lane) were fractionated on a 1.0% agarose gel containing 2.2M formaldehyde and then transferred to a

Hybond-N⁺ filter (Amersham, Buckinghamshire, UK). The filter membranes were hybridized at 42°C using the probe in a solution containing 5×SSC, 50% formamide, 1×Denhart's, 20mM sodium phosphate (pH 6.8), 5mM EDTA, 0.2% SDS and 100 µg/ml sheared salmon sperm DNA. The probes were labeled with [α -³²P]dCTP using a random priming reaction and were hybridized under the conditions described above. Thereafter, the blots were rehybridized with the GAPDH probe as a control for RNA loading. Autoradiographs were analyzed by densitometry (ImageQuat, Molecular Dynamics).

Determination of DNA synthesis. One milliliter of cell suspension containing 5×10⁴ cells was seeded in each well of 24-well plates and incubated for 24 h. The medium was then replaced with 0.2% FBS-MEM, and cultures were incubated for 3 days in order to arrest the cells at the G₀-G₁ phase. DNA synthesis was determined by labeling the cells with [³H]thymidine (5 µCi/ml) for 1 h starting 0 to 36 h after the re-addition of serum. DNA synthesis is expressed as dpm incorporated [³H]thymidine per µg of cellular protein.

Results and Discussion

Cloning of a cell-crisis gene. The clone obtained encoded a protein containing 130 amino acids and a helix-loop-helix (HLH) domain according to analysis with GeneWorks software (IntelliGenetics, Inc., CA, USA) (Fig. 1). HLH proteins define a class of transcription factors, and HLH regions, which consist of two amphipathic α helices separated by a loop of variable length and sequence, is required for dimerization (10). It is well known that these HLH transcription factors play an important role in the progression of the cell cycle and cell differentiation and development. This gene was named C-1 and submitted to the GenBank data library under accession number U41816.

The sequence of C-1 (nucleotide 54-429) had 93% homology with part of the human HepG2 3'-directed *Mbo* I cDNA clone s14d04 (HUM0S14D04, accession number D12019, nucleotide 1-376) (11). C-1 gene and the clone s14d04 may be the same gene, although C-1 appears to contain a full-length coding region (a 5' coding region and 5' non-coding region in addition to the homologous region of the s14d04), whereas the clone s14d04 is an expressed sequence tag and contains no information of open reading frame (ORF) and poly(A)

TG GCG GCC ACC ATG AAG AAG GCG GCT GCA GAA GAT GTC AAT GTT ACT	47
<u>M</u> <u>K</u> <u>K</u> <u>A</u> <u>A</u> <u>A</u> <u>E</u> <u>D</u> <u>V</u> <u>N</u> <u>V</u> (T)	(12)
TTC GAA GAT CAA CAA AAG ATA AAC AAA TTT GCA CGG AAT ACA AGT AGA	95
E E D Q Q K I N K F A R N <u>[T]</u> <u>S</u> <u>B</u>	(28)
ATC ACA GAG CTG AAG GAA GAA ATA GAA GTA AAA AAG AAA CAA CTC CAA	143
<u>I</u> <u>T</u> <u>E</u> <u>L</u> <u>K</u> <u>E</u> <u>E</u> <u>I</u> <u>E</u> <u>V</u> <u>K</u> <u>K</u> <u>K</u> <u>Q</u> <u>L</u> <u>Q</u>	(44)
AAC CTA GAA GAT GCT TGT GAT GAC ATC ATG CTT GCA GAT GAT GAT TGC	191
<u>N</u> <u>L</u> <u>E</u> <u>D</u> <u>A</u> <u>C</u> <u>D</u> <u>D</u> <u>L</u> <u>M</u> <u>L</u> <u>A</u> <u>D</u> <u>D</u> <u>D</u> <u>C</u>	(60)
TTA ATG ATA CCT TAT CAA ATT GGT GAT GTC TTC ATT AGC CAT TCT CAA	239
L M I P Y Q I G D V F I S H <u>(S)</u> <u>Q</u>	(76)
GAA GAA ACG CAA GAA ATG TTA GAA GAA GCA AAG AAA AAT TTG CAA GAA	287
<u>E</u> <u>E</u> <u>T</u> <u>Q</u> <u>E</u> <u>M</u> <u>L</u> <u>E</u> <u>E</u> <u>A</u> <u>K</u> <u>K</u> <u>N</u> <u>L</u> <u>Q</u> <u>E</u>	(92)
GAA ATT GAC GCC TTA GAA TCC AGA GTG GAA TCA ATT CAG CGA GTG TTA	335
<u>E</u> <u>L</u> <u>D</u> <u>A</u> <u>L</u> <u>E</u> <u>(S)</u> <u>B</u> <u>V</u> <u>E</u> <u>S</u> <u>I</u> <u>Q</u> <u>R</u> <u>V</u> <u>L</u>	(108)
GCA GAT TTG AAA GTT CAG TTG TAT GCA AAA TTC GGG AGC AAC ATA AAC	383
A D L K V Q L Y A K F G S N <u>I</u> <u>N</u>	(124)
CTT GAA GCT GAT GAA AGT TAA ACA TTT TAT AAT ACT TTT TTT ATT TGT	431
<u>L</u> <u>E</u> <u>A</u> <u>D</u> <u>E</u> <u>S</u> *	(130)
TTA ATA AAC TTG AAT ATT GTT TAA AAT GAT AAT TTC CTT CTT CAA ATG	479
ACA TGG AAA GCA AAA CTT TCT TTT TTA AAA ATT TTC ATT TAT TTA ATG	527
GAA ACT TGC CCA TTT TCA CAT GTC TGC TTA TTT ATT TTA TAT TTT TAA	575
AAG AAG ACA GTA TTC ACC TAT GTA TTT TGC ATA ACG ATT ATA TCA AGT	623
CTA GGG GCT TCA TGT CAT GTT ATT AAA ATC AGT TAA GCA ATC TTT TAT	671
GTT TCT ATA TTA TTT AGA ATA TTT GTT GTT GCA ATT TTC ACA TAA GAA	719
AAT TTA ACA GTT GTG TCA TGT TGT TTC TGT CTG ATT TTA ATT GCT GTC	767
TAA TGA CGG GGA AAG CAC GAC GAA AAG ATG TAC AAT CCT GCA TCC TTG	815
CTT ATT TCA CAA CTA AAG CTT TGT CAT AGA CTT CAA AAT ATA TAT GTA	863
TAT ATT TTA TTT AAA TAT ATG TTA CAT ATT ATA TTT AAA CAT ACA TAT	911
TTA ACA TTT TTT ACA TAT CTA TCA ATA TCA GAG ATT TGG GTA AAA GAA	959
TGG GTA ATG TTT AAA CAT GTG GAG GCA TGT GGA GCT TTA TAC AAA CAG	1007
GGC AGA ACC ACA GAA GAC GTT TTA GAA ACC AAG AGA TGT GCA GAA AGA	1055
ATG TTT AGT GTT TTT TCG TTT TAA ATT TTA GAT TTT ATT TTA GTG CTT	1103
TGT AAT TAA TTG GGG TTT ATA TTG ATA AAG ATG TGG AAG TTA AAC AGC	1151
TAT GTA TGT AAA AGT AAG <u>GCT TAT TTC</u> TTA AAT AAA GGA TGC ATT TCT	1199
TCC CAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA	1241

Fig. 1 Nucleotide and amino acid sequences of human C-1 cDNA. The predicted amino acid sequence is shown below the nucleotide sequence, using the standard single-letter amino acid code. The poly(A) signal is double-underlined (nt 1182-1187). Possible alpha-helix-forming regions are indicated by the broken line (aa 1-10, 26-60, 75-101 and 123-130). Consensus sites phosphorylated by protein kinase C and casein kinase II are shown by square and round brackets, respectively.

signal. However, we could not rule out the possibility that the C-1 gene and the clone s14d04 belong to the same family. The C-1 protein is about 40 % homologous to the yeast protein that is similar to both the human myotonic dystrophy kinase (N1761, accession number X92517) and to the 3' end of human skeletal muscle β -topomyosin mRNA (TPM2, accession number M12126).

mRNA expression during the cell cycle of

immortal human fibroblast cell lines and their normal parent cells. Serum starvation was used to synchronize the cell cycle. Under these conditions, DNA synthesis (measured by [³H]thymidine incorporation) began 12-18h after the re-addition of serum and reached a maximum after about 24h (Fig. 2A), whereas the immortal cells showed some DNA replication even during serum starvation.

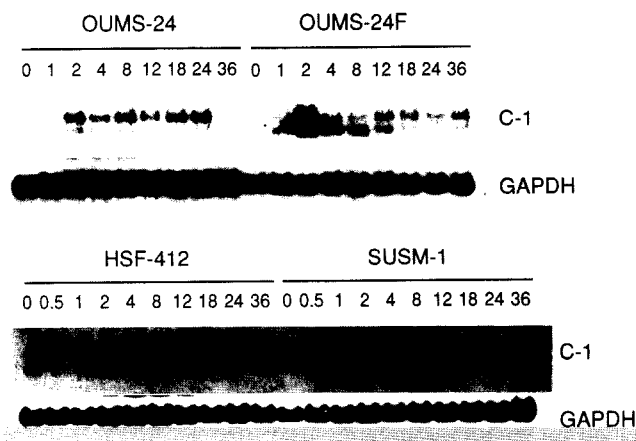
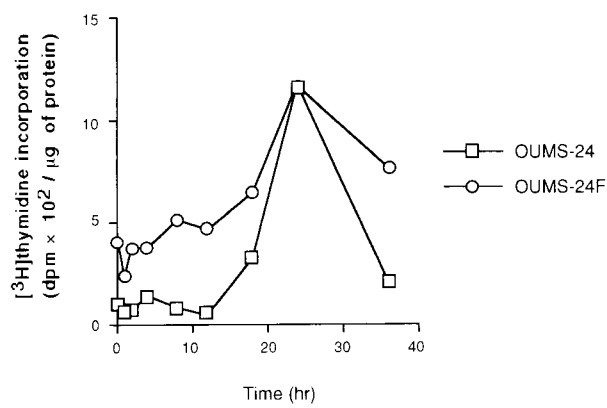


Fig. 2 Serum-stimulated induction of C-1 mRNA in immortalized human fibroblasts and their normal parent cells. Subconfluent cultures of the immortal cells (OUMS-24F and SUSM-1) and their normal parent cells (OUMS-24 and HSF-412) were maintained in MEM + 0.2% FBS for 3 days and were then stimulated with MEM + 10% FBS at time zero. (A) At the times indicated, cells were labeled with $5 \mu\text{Ci/ml}$ [^3H] thymidine for 1 h, and the radioactivity of the acid-insoluble fraction was measured. (B) Total RNA was isolated at the indicated times, and $15 \mu\text{g}$ of RNA was loaded per lane, fractionated by electrophoresis and then transferred to a Hybond-N⁻ filter. The expression of C-1 mRNA was detected by Northern blot analysis using C-1 clone as a probe. This probe recognized two C-1 mRNAs of about 0.6 and 0.8 kb. (C) Quantitation of radioactivity in the individual bands in Fig. 2B was done by scanning the blots by densitometry. The ratio of C-1/GAPDH intensity was plotted for the given times after serum stimulation.

Then the expression level of C-1 mRNA was examined at different stages of the cell cycle by Northern blot analysis (Fig. 2B). Only small amounts of C-1 message were detected while cells were in the G₀ phase. Maximum expression of C-1 message occurred in the early G₁ phase, 2 h after serum was re-added to the OUMS-24F, OUMS-24 and HSF-412 cultures (Fig. 2C). These results indicate that the expression of the C-1 gene is transiently induced in the early part of the G₁ transition in these cells. In the case of SUSM-1 cells,

high expression of C-1 was observed in the middle part of the G₁ phase. This rather delayed expression may be due to the fact that the immortalized cells did not completely stop proliferation under serum-free culture conditions.

Northern blotting of RNA revealed that C-1 transcripts were approximately 0.6 and 0.8 kb in length, though C-1 cDNA had 1261 nucleotides (nt). This discrepancy may be due to the artificial ligation of the library construction, because the other poly (A) signal exists at nt 434-439. Otherwise, the 0.6- and 0.8-kb transcripts

may be derived by alternative RNA splicing or from a family gene.

We intended to clone cell-crisis genes from the cells at the crisis stage, because these genes may be overexpressed in the crisis cells and associated with cell death. However, expression of the cloned C-1 gene was greater in the immortalized actively proliferating cells than in the normal cells. This indicates that the C-1 gene does not cause cell death. Since it is well known that dying and actively proliferating cells coexist in the crisis cultures treated with SV40, the C-1 gene was probably cloned from the proliferating cells rather than the dying cells at the crisis stage. In conclusion, it seems that the C-1 gene product functions as a transcription factor related to the cell cycle and may contribute to cell immortalization.

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Received December 13, 1995; accepted February 1, 1996.