Jpn. J. Cancer Res. **87**, 58–61, January 1996

Cloning of Mouse *DAN* cDNA and Its Down-regulation in Transformed Cells

Toshinori Ozaki,[1] Jun Ma,[1] Keizo Takenaga[2] and Shigeru Sakiyama[1,3]

[1]*Division of Biochemistry and* [2]*Division of Chemotherapy, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuo-ku, Chiba 260*

Recently, we have demonstrated that *DAN* gene product exhibits a tumor-suppressive activity *in vitro*. We report here the cloning and sequencing of a mouse *DAN* cDNA that contains the entire coding region. Sequence analysis revealed that mouse *DAN* cDNA is 1691 nucleotides in length and contains an open reading frame of 178 amino acids. The deduced mouse DAN protein sequence shows 96% and 93% identity with the counterparts isolated from rat 3Y1 fibroblasts and normal human lung, respectively. Genomic Southern blot hybridization indicated that *DAN* gene exists as a single copy in the mouse genome. The expression of *DAN* gene was suppressed in a variety of transformed NIH3T3 cells when compared with that in the parental NIH3T3 cells.

Key words: Down-regulation — *DAN* gene — NIH3T3 cell line

Various types of candidate tumor-suppressor genes have been cloned by means of a differential screening strategy,[1–6] based on the concept that the expression of tumor-suppressor genes might be reduced or absent in malignant cells.[7] *DAN* gene was originally isolated from a rat fibroblast 3Y1 cDNA library by using this approach.[8] The expression of *DAN* gene is significantly decreased in a variety of transformed 3Y1 cells, compared with that in the parental 3Y1 cells.[8] Interestingly, the overexpression of *DAN* gene product in v-*src*-transformed 3Y1 cells causes a marked decrease of the transformed phenotype, suggesting that *DAN* gene product contains a tumor-suppressive activity.[9] Recent work in our laboratory demonstrated that human *DAN* gene is localized at 1p36.11–p36.13, which resides within the commonly deleted chromosome region in neuroblastoma, and allelic abnormalities within *DAN* gene locus have been observed in several neuroblastoma cases.[10] Thus, human *DAN* gene might be a candidate for a tumor suppressor gene of human neuroblastoma.

As the functional analysis of *DAN* gene product was carried out only in rat fibroblasts, it is important to clarify the biological role(s) of *DAN* gene product in other cell systems. As a first step, we isolated mouse *DAN* cDNA from a NIH3T3 cDNA library. Here we report the sequence of mouse *DAN* cDNA and the expression of *DAN* gene in various types of transformed NIH3T3 cells.

MATERIALS AND METHODS

Culture conditions All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere.

Screening of a cDNA library An NIH3T3 cDNA library (approximately 7×10^5 recombinants) was screened with ^{32}P-labeled Δ41PE DNA.[11] The Δ41PE DNA contains the 5′-portion of the translated region of rat *DAN* cDNA.[10] Hybridization was performed in a solution containing 50% formamide, 6×SSC (1×SSC; 0.15 *M* NaCl, 15 m*M* sodium citrate), 5×Denhardt's solution[12] and 0.1% sodium dodecyl sulfate (SDS) at 42°C overnight. The filters were washed at 50°C for 30 min to a final stringency of 0.1×SSC containing 0.1% SDS.[13]

DNA sequencing Mouse *DAN* cDNA was digested with appropriate restriction enzymes and subcloned into pTZ18R (Pharmacia, Uppsala, Sweden). The plasmid DNA was subjected to sequencing by the dideoxynucleotide chain-termination procedure[14] using Sequenase (United State Biochemicals, Cleveland, Ohio).

Southern blot analysis High-molecular-weight genomic DNA was digested completely with appropriate restriction enzymes and size-fractionated in 0.8% agarose gel. The DNA was transferred to a nylon membrane filter, which then was hybridized overnight at 65°C in 0.9 *M* NaCl, 0.05 *M* phosphate, pH 7.4, 5 m*M* EDTA, 5× Denhardt's solution, 0.2% SDS, 10% dextran sulfate, and 200 μg of heat-denatured salmon sperm DNA per ml containing ^{32}P-labeled mouse *DAN* cDNA. The filter was rinsed in 1×SSC containing 0.2% SDS at 65°C for 30 min.[13]

Northern hybridization Total RNA (10 μg) was electrophoresed in 1% agarose/formaldehyde gel, transferred to a nylon membrane filter, and immobilized by UV crosslinking. Hybridization was carried out in the same manner as for Southern blot analysis.

Nucleotide sequence accession number The nucleotide sequence data reported in this work have been submitted

[3] To whom correspondence should be addressed.

to DDBJ/GenBank/EMBL and assigned the accession number D50263.

RESULTS AND DISCUSSION

In order to obtain a full-length mouse *DAN* cDNA clone, we have screened approximately 7×10^5 phage recombinants from an oligo-dT primed cDNA library of NIH3T3 cells. Under the standard conditions with radiolabeled Δ41PE DNA, which contains a 5′-portion of the translated region of rat *DAN* cDNA, we have obtained eight positive clones. Among them, clone R6 contained the largest cDNA insert (ca. 1.8 kb) based on restriction analysis. As the size of this cDNA insert was almost the same as the estimated size of *DAN* transcript by Northern blot hybridization (see below), we tentatively concluded that it was close to full length and determined its nucleotide sequence. The restriction map and the complete nucleotide sequence of R6 are shown in Fig. 1. The R6 is 1691 nucleotides in length and contains a polyadenylation signal (AATAAA) 22 bp upstream of the poly(A) tail at the 3′-terminus. A significant sequence homology was detected between R6 and *DAN* cDNAs of rat and human.[8, 10] Overall, the nucleotide sequence homology of R6 with rat and human *DAN* cDNAs was 88% and 66%, respectively. Therefore, we concluded that R6 does represent the mouse counterpart of *DAN* cDNA.

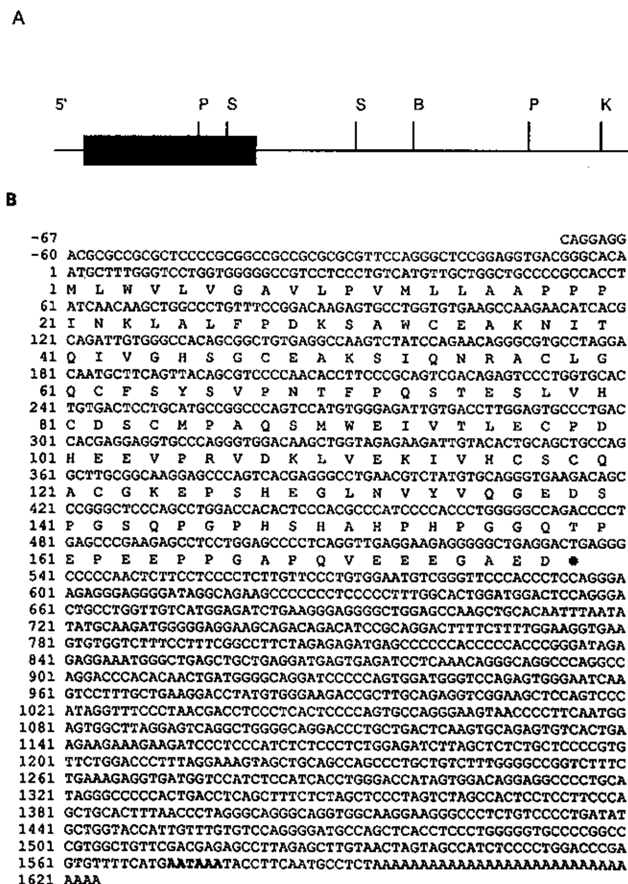

Fig. 1. (A) Restriction map of mouse *DAN* cDNA. The thin line shows the 5′- or 3′-noncoding region; the bold line indicates the coding region. B, *Bam*HI; K, *Kpn*I; P, *Pst*I; S, *Sma*I. (B) Nucleotide and deduced amino acid sequences. The nucleotides and amino acids are numbered from the beginning of the translation initiation site (ATG). The stop codon (TGA) is shown by an asterisk and the polyadenylation signal is indicated by bold characters.

There exists a single open reading frame (ORF) extending from nucleotide 1 to a stop codon at position 534. The deduced amino acid sequence of mouse DAN protein consists of 178 residues with a predicted molecular weight of 19164.27 Da. Its validity was confirmed by *in vitro* transcription and translation experiments (data not shown). A comparison of the amino acid sequence of mouse DAN protein with the rat and human counterparts is shown in Fig. 2. Overall, there is 96% sequence identity between mouse and rat and 93% between mouse and human.[8, 10] Mouse DAN protein includes several structural features which are also found in DAN protein of other species. It contains casein kinase II target sites at residues 46–49 and 89–92, an N-linked glycosylation site at residues 38–40, a zinc finger-like structure at residues 81–101 and a proline-rich sequence in the C-terminal region at residues 141–171 (Fig. 2). Considering the extremely high sequence homology, *DAN* gene product might have a biologically crucial function in mammals.

In order to investigate the genomic organization of mouse *DAN* gene, genomic DNA from NIH3T3 cells was digested with various kinds of restriction enzymes and hybridized with the radio-labeled full-length mouse *DAN* cDNA. As shown in Fig. 3, the hybridization pattern was quite simple, which indicates that *DAN* gene is present as a single copy in the mouse genome, as observed in other species.[8)]

As the expression of *DAN* gene was significantly suppressed in a variety of transformed rat 3Y1 fibroblasts,[8)] it is important to investigate whether this phenomenon is common to other cell systems. Total RNA was isolated from the following cell lines, *erb*B2-, v-*src*-, c-Ha-*ras*-, v-Ki-*ras*-, v-Ha-*ras*- and N-*ras*-transformed NIH3T3 cells, and *DAN* mRNA levels of the cell lines were analyzed by Northern hybridization (Fig. 4). In NIH3T3 cells, mRNA of about 2 kb in length was detected, its size being almost the same as that of the rat counterpart. A marked decrease in *DAN* mRNA level was detected in all the transformed cell lines except in N-*ras*-transformed cells. These observations suggest that the negative regulation of *DAN* gene expression is not specific to a rat cell

```
MOUSE  (1)   MLWVLVGAVL PVMLLAAPPP INKLALFPDK SAWCEAKNIT QIVGHSGCEA
RAT    (1)   MLWVLVGTVL PVMLLAAPPP INKLALFPDK SAWCEAKNIT QIVGHSGCEA
HUMAN  (1)   MLRVLVGAVL PAMLLAAPPP INKLALFPDK SAWCEAKNIT QIVGHSGCEA

             KSIQNRACLG QCFSYSVPNT FPQSTESLVH CDSCMPAQSM WEIVTLECPD
             KSIQNRACLG QCFSYSVPNT FPQSTESLVH CDSCMPAQSM WEIVTLECPG
             KSIQNRACLG QCFSYSVPNT FPQSTESLVH CDSCMPAQSM WEIVTLECPG

             HEEVPRVDKL VEKIVHCSCQ ACGKEPSHEG LNVYVVQGEDS PGSQPG--PH
             HEEVPRVDKL VEKIVHCSCQ ACGKEPSHEG LNVYMQGEDG PGSQPG--SH
             HEEVPRVDKL VEKILHCSCQ ACGKEPSHEG LSVYVQGEDG PGSQPGTHPH

             SHAHPHPGGQ TPEPEEPPGA PQVEEEGAED (178)
             SHSHPHPGCQ TPEPEEPPGA PQVEEEGAED (178)
             PHPHPHPGGQ TPEPEDPPGA PHTEEEGAED (180)
```

Fig. 2. Amino acid sequence comparison among mammalian DAN proteins (mouse, rat and human). Shaded boxes indicate identical amino acids among them. Putative functional domains are as follows; an N-linked glycosylation site at 38–40 (wavy lines), two casein kinase II target sites at 46–49 and 89–92 (filled circles), a zinc finger-like structure at 81–101 (dashed lines) and a proline-rich sequence at 141–171 (proline residues are indicated by underlines).

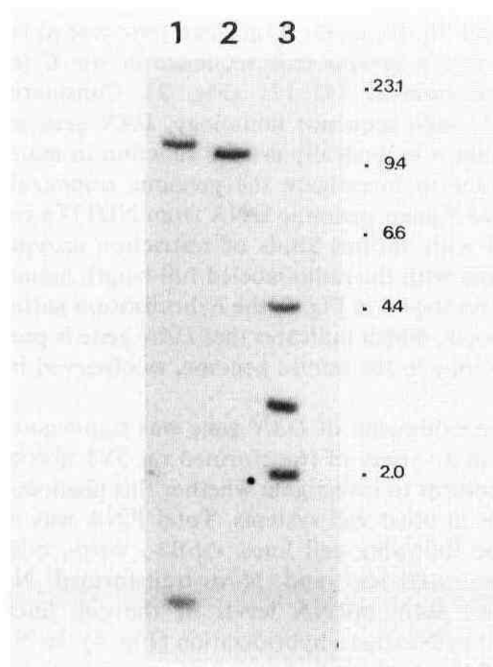

Fig. 3. Southern blot analysis of NIH3T3 genome. High-molecular-weight genomic DNA from NIH3T3 cells (10 μg/lane) was digested with *Bam*HI (lane 1), *Eco*RI (lane 2) or *Xba*I (lane 3), separated in a 0.8% agarose gel, transferred to a nylon membrane and hybridized with radio-labeled full-length mouse *DAN* cDNA. Sizes are shown in kilobases.

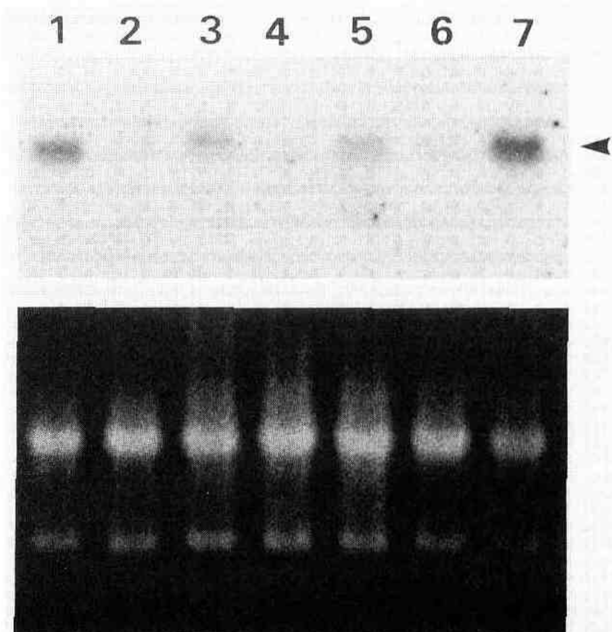

Fig. 4. *DAN* gene expression in various types of transformed NIH3T3 cells. Total RNA (10 μg) derived from NIH3T3 (lane 1), *erb*B2- (lane 2), v-*src*- (lane 3), c-Ha-*ras*- (lane 4), v-Ki-*ras*- (lane 5), v-Ha-*ras*- (lane 6) and N-*ras*-transformed NIH3T3 cells (lane 7) was electrophoresed in a 1% agarose/formaldehyde gel, blotted onto a nylon membrane and probed with ^{32}P-labeled full-length mouse *DAN* cDNA. The lower panel indicates the ethidium bromide-stained gel prior to blotting.

system. We have previously shown that the expression of *DAN* gene was not decreased in v-Ha-*ras*-transformed 3Y1 cells.[8] This is contrary to the present result and the reason for this discrepancy between these two closely related species is unknown.

Contente *et al.* have reported that the expression of *rrg*, which possesses a tumor-suppressive activity identified in NIH3T3 cell systems, is down-regulated in several types of transformed NIH3T3 cells including v-Ki-*ras*-,c-Ha-*ras*- and v-Ha-*ras*-transformed cells.[3] As described previously, rat *DAN* gene product, when overexpressed, exhibits a tumor-suppressive activity in v-*src*-transformed 3Y1 cells.[9] Although the precise functions of DAN protein are still obscure, in spite of its rather ubiquitous expression,[8] it has been demonstrated that the overexpression of rat DAN protein in 3Y1 cells results in retardation of entry into the S phase.[15] Due to the very similar characteristics of rat and mouse *DAN* genes, it is reasonable to speculate that *DAN* gene product also participates in the negative regulatory mechanisms of cellular growth and/or transformation of mouse cell systems.

ACKNOWLEDGEMENTS

We thank Dr. T. Yamamoto (Laboratory of Oncology, Institute of Medical Science, University of Tokyo) for supplying v-*src*- and *erb*B2-transformed cells and Dr. Y. Yuasa (Department of Hygienics, Tokyo Medical and Dental University) for v-Ki-*ras*- and N-*ras*-transformed cells. We are grateful to Dr. H. Enomoto (Division of Biochemistry, Chiba Cancer Center Research Institute) for valuable discussions. This work was supported by a Grant-in-Aid for the Second-term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare, Japan, and by a grant from the Uehara Memorial Foundation.

(Received September 6, 1995/Accepted October 16, 1995)

REFERENCES

1) Pachnis, V., Belayew, A. and Tilghman, S. M. Locus unlinked to α-fetoprotein under the control of the murine *raf* and *Rif* genes. *Proc. Natl. Acad. Sci. USA*, **81**, 5523–5527 (1984).
2) Steeg, P. S., Bevilacqua, G., Kopper, L., Thorgeirsson, U. P., Talmadge, J. E., Liotta, L. A. and Sobel, M. E. Evidence for a novel gene associated with low tumor metastatic potential. *J. Natl. Cancer Inst.*, **80**, 200–204 (1988).
3) Contente, S., Kenyon, K., Rimoldi, D. and Friedman, R. M. Expression of gene *rrg* is associated with reversion of NIH3T3 transformed by LTR-c-H-*ras*. *Science*, **249**, 796–798 (1990).
4) Dowdy, S. F., Lai, K.-M., Weissman, B. E., Matsui, Y., Hogan, B. L. M. and Stanbridge, E. J. The isolation and characterization of a novel cDNA demonstrating an altered mRNA level in nontumorigenic Wilms' microcell hybrid cells. *Nucleic Acids Res.*, **19**, 5763–5769 (1991).
5) El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825 (1993).
6) Zou, Z., Anisowicz, A., Hendrix, M. J. C., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E. and Sager, R. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. *Science*, **263**, 526–529 (1994).
7) Lee, S. W., Tomasetto, C. and Sager, R. Positive selection of candidate tumor-suppressor genes by subtractive hybridization. *Proc. Natl. Acad. Sci. USA*, **88**, 2825–2829 (1991).
8) Ozaki, T. and Sakiyama, S. Molecular cloning and characterization of a cDNA showing negative regulation in v-*src*-transformed 3Y1 rat fibroblasts. *Proc. Natl. Acad. Sci. USA*, **90**, 2593–2597 (1993).
9) Ozaki, T. and Sakiyama, S. Tumor-suppressive activity of *N03* gene product in v-*src*-transformed rat 3Y1 fibroblasts. *Cancer Res.*, **54**, 646–648 (1994).
10) Enomoto, H., Ozaki, T., Takahashi, E., Nomura, N., Tabata, S., Takahashi, H., Ohnuma, N., Tanabe, M., Iwai, J., Yoshida, H., Matsunaga, T. and Sakiyama, S. Identification of human *DAN* gene, mapping to the putative neuroblastoma tumor suppressor locus. *Oncogene*, **9**, 2785–2791 (1994).
11) Feinberg, A. P. and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, **132**, 6–13 (1984).
12) Denhardt, D. A membrane filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.*, **23**, 640–646 (1966).
13) Sambrook, J., Fritsch, E. F. and Maniatis, T. "Molecular Cloning: A Laboratory Manual" (1989). Cold Spring Harbor Laboratory Press, New York.
14) Sanger, F., Nicklen, S. and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467 (1977).
15) Ozaki, T., Nakamura, Y., Enomoto, H., Hirose, M. and Sakiyama, S. Overexpression of *DAN* gene product in normal rat fibroblasts causes a retardation of the entry into the S phase. *Cancer Res.*, **55**, 895–900 (1995)