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A *REIC* Gene Shows Down-Regulation in Human Immortalized Cells and Human Tumor-Derived Cell Lines

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Normal human cells stop proliferation after a certain number of cell divisions. This phenomenon is called cellular aging. The fact that the senescence phenotype is dominant and the immortal one is recessive indicates that immortalization of human cells may be caused by loss of functions of certain genes in normal cells. Based on this evidence, several cDNA clones whose expression was down-regulated during the immortalization process of human cells were isolated by the representative difference analysis (RDA) system in our laboratory. One of them, which was named REIC, was expressed to a lower degree in three human immortalized cell lines as compared with their parental normal counterparts. In addition, the expression of REIC was markedly lower in eight human tumor-derived cell lines (Hep3B and HuH-7 hepatocellular carcinomas, HuH-6 Clone 5 hepatoblastoma, HuCCT-1 cholangiocarcinoma, A549 lung cancer, HaCaT immortalized keratinocyte, HeLa cervical carcinoma, and Saos-2 osteosarcoma). In contrast, among the human tissues examined, the heart and brain, which contain a large number of post-mitotic cells, showed the highest expression of REIC. The full-length REIC cDNA revealed that the predicted protein is 350 amino acids in length and possesses coiled-coil tertiary structures in each of the amino- and carboxyl-termini. Furthermore, a search of the protein database revealed a match of this gene product with Dkk-3, which is a novel inhibitor of Wnt oncogene. These results indicate that the REIC cloned by us may function as a tumor suppressor. **© 2000 Academic Press**

Normal human cells enter the irreversible growth-arrested state after limited divisions in culture, which is defined as cellular senescence [1], and escape from cellular senescence results in immortalization of cells [2]. Once normal human cells are immortalized, they are relatively easily transformed into neoplastic cells with carcinogenic agents such as chemical carcinogens, physical agents and so-called oncogenes [2, 3]. These facts indicate that the immortalization of cells is a critical step for neoplasmic transformation of human cells.

Since hybrid cells obtained by fusion between normal and immortal cells exhibit limited division potential, the normal phenotype is supposed to be dominant and the immortal phenotype to be recessive [4]. Moreover, introduction of certain human chromosomes (#1, 4, 6, and 7) into various human immortalized cells induces cellular senescence. These findings indicate that immortalization can be caused by loss or dysfunction of certain genes [5–8]. In fact, immortalization-related genes such as *HIC-5* [9], *ING1* [10], *MORF-4* [11] and *ST-7* [12] have been identified. Recently, other genes, *SEN 6A, SEN6* and *SEN16,* have been reported to induce cellular senescence [13–15].

We previously succeeded in establishing three human immortalized cell lines by treatment with either ^{60}Co γ-rays or a chemical carcinogen, 4-nitroquinoline 1-oxide [2]. These immortalized cell lines (KMST-6, SUSM-1, and OUMS-24F) continue to grow without showing cellular senescence, whereas their normal counterparts (KMS-6, HSF-412, and OUMS-24, respectively) become senescent. Therefore, we attempted to isolate the immortalization-related genes using these immortalized cell lines and their normal counterparts. We used an RDA system that utilized the difference in gene expression between a human immortalized KMST-6 cell line and its parental normal KMS-6 cell strain. In this paper, we report the cloning and characterization of a REIC cDNA (Reduced Expression in Immortalized Cells) whose expression was markedly decreased in human immortalized cells and human tumor-derived cell lines. Furthermore, we discuss the relationship between down-regulation of this gene and immortalization of human cells.

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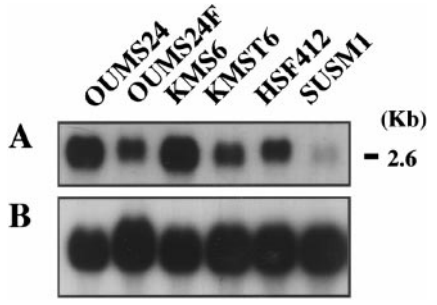

FIG. 1. Decreased expression of REIC mRNA in human immortalized cell lines as compared to their normal counterparts. (A) Total RNA (10 μg) was fractionated by electrophoresis and hybridized with ^{32}P-labeled cDNA for REIC, using a cloned REIC DNA fragment as a probe. (B) The blot was rehybridized with a probe for β-actin as a loading control.

MATERIALS AND METHODS

Cell culture. Normal human diploid fibroblasts (KMS-6 and OUMS-24) and their immortal counterparts (KMST-6 and OUMS-24F) were used. The latter cells were immortalized from the former cells by repeated treatments with ^{60}Co-γ rays and a chemical carcinogen, respectively [16, 17]. Cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, Intergen, NY), 2 mM glutamine and 100 μg/ml kanamycin. Human tumor-derived cells, Hep3B and HuH-7 hepatocellular carcinomas, HuH-6 Clone 5 hepatoblastoma, HuCCT-1 cholangiocarcinoma, A549 lung cancer, HaCaT immortalized keratinocytes, HeLa cervical carcinoma, Saos-2 osteosarcoma, and T24 bladder carcinoma, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 100 μg/ml kanamycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and subcultured with 0.2% trypsin plus 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) when they reached confluence.

Representative difference analysis. Total cellular RNA was extracted by the guanidinium thiocyanate-phenol method, and poly $(A)^+$ RNA was purified from total RNA (0.5 mg) by oligo (dT) beads (DYNABEADS, Oslo, Norway). Two kinds of complimentary DNA (cDNA) pools were synthesized from poly $(A)^+$ RNA (2 μg) derived from senesced KMS-6 (48 PDLs) and immortalized KMST-6 (358 PDLs). Suppression subtractive hybridization was performed using a PCR-Select cDNA subtraction kit according to the manufacturer's protocol (CLONTECH, CA). Under these conditions, subtracted PCR products were passed through CHROMA SPIN-100 (CLONTECH, CA) to remove fragments less than 100 bp. The remaining cDNAs were subcloned into pT7Blue TA-cloning vectors (Novagen, WI).

Northern blot analysis. Total cellular RNA was extracted as described above. Total cellular RNA (10 μg) was fractionated by electrophoresis through a formaldehyde-agarose gel and transferred to a Hybond N^+ membrane. The membrane was hybridized at 42°C with a probe in solution containing 5× SSC, 50% formamide, 1× Denhart's solution, 20 mM sodium phosphate (pH 6.8), 5 mM EDTA, 0.2% SDS and 100 μg/ml sheared heat-denatured salmon sperm DNA. The cDNA probe was labeled with [α-^{32}P]dCTP by a random priming reaction.

Isolation of full-length REIC cDNA. In order to obtain full-length REIC cDNA, we screened a human heart cDNA library (Gibco BRL, MD) using a partial REIC sequence obtained from the RDA system as a probe. An additional 5′ sequence of this gene, which contains the 5′-cap structure, was isolated by the 5′ rapid amplification of cDNA ends (5′-RACE) method using a human heart Cap Site cDNA library (Nippon Gene, Toyama, Japan) with the REIC gene-specific primers (5′-GTCCTCCATCAGTTCCTCAAC-3′, 5′-CTTGACTGGAGCCGAGGTCG-3′, and 5′-CAAGCCGCTGCATTTCCGCTCTG-3′).

RESULTS

Using the RDA system we obtained a cDNA fragment (D93 clone) whose expression was lower in the immortalized KMST-6 cell line as compared with its parental normal counterpart. Expression of the D93 clone was also down-regulated in two other human immortalized cell lines but was not in their parental normal counterparts (Fig. 1). Northern blot analysis showed two specific transcripts, a major 2.6-kb transcript and a minor 8-kb one. The expression of the 2.6-kb transcript was markedly reduced in eight human tumor-derived cell lines (Hep3B and HuH-7 hepatocellular carcinomas, HuH-6 Clone 5 hepatoblastoma, HuCCT-1 cholangiocarcinoma, A549 lung cancer, HaCaT immortalized keratinocytes, HeLa cervical carcinoma, and Saos-2 osteosarcoma) (Fig. 2). Only human bladder carcinoma T24 cell line expressed it nearly at the same level as normal human fibroblasts. These results indicate that expression of the D93 clone is down-regulated in many kinds of human cancer cell lines, suggesting that its gene product may play a role in tumor suppression.

Among the human tissues examined, the 2.6-kb transcript was highly expressed in the heart and brain, which contain a large number of post-mitotic cells; moderately expressed in the skeletal muscle, colon, spleen, kidney, liver, small intestine, placenta and lung; and barely detectable in the thymus and peripheral blood leukocytes (Fig. 3). On the other hand, the high molecular weight transcript (8-kb) was highly expressed in the liver and placenta.

Then we isolated a full-length D93 clone. By screening a human heart cDNA library, we obtained eight cDNA clones with inserts from 1.0–2.5 kb, corresponding to the D93 clone. Although the insert of the longest

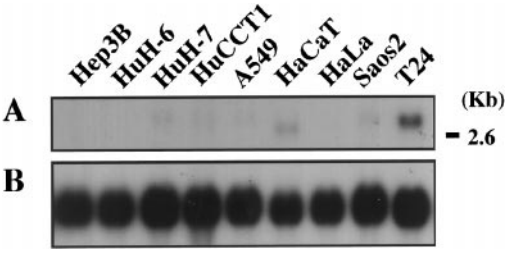

FIG. 2. Northern blot analysis of REIC expression in human tumor-derived cell lines. (A) Total RNA (10 μg), obtained from various cancer cell lines, was fractionated by electrophoresis and hybridized with ^{32}P-labeled REIC cDNA. (B) The blot was rehybridized with a probe for β-actin as a loading control.

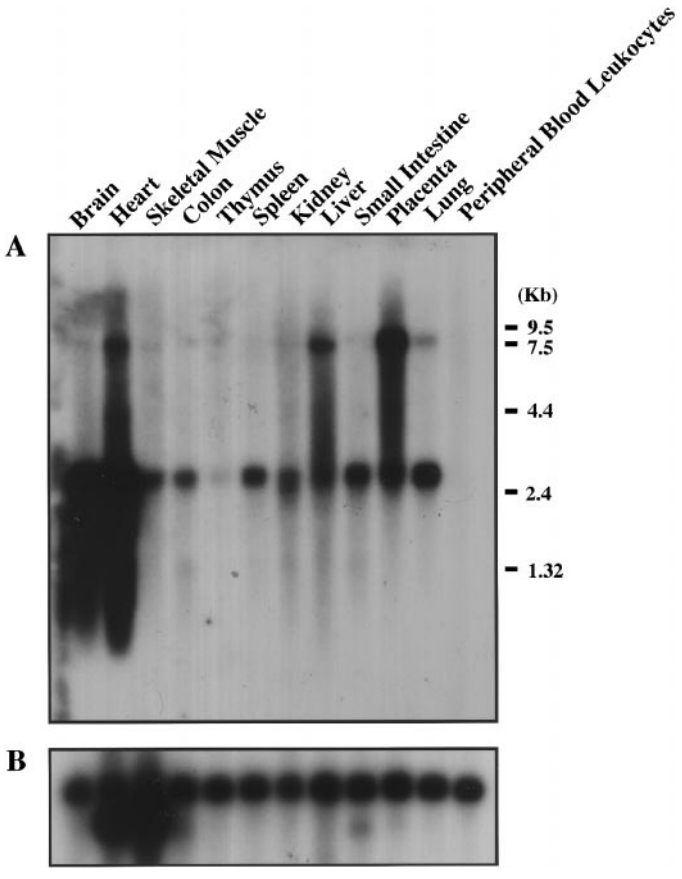

FIG. 3. Expression of the *REIC* gene in various normal human tissues. Human multiple tissue blots (Clontech) containing poly $(A)^+$ RNA (2 μg) from indicated tissues were probed with ^{32}P-labeled REIC cDNA fragments. (B) The blot was rehybridized with a probe for β-actin as a loading control.

clone (approximately 2.5-kb) was almost the same as the size of a major band observed in Northern blotting (Fig. 1), it did not have an in-frame stop codon in its 5′-upstream region. Therefore, we carried out 5′-oligo cap cloning to recover the complete 5′-end of fragment D93 and obtained full-length cDNA of D93 clones containing a 5′-cap structure (Fig. 4). This newly cloned gene was named REIC (Accession number; AB034203).

The nucleotide sequence (GAAATGC) neighboring the first ATG codon resembles the context of the Kozak consensus initiation site of eukaryotic mRNA translation [18]. An open reading frame (ORF) extends from 198–1247 and encodes a protein of 350 amino acids with an estimated molecular weight of 38.3 kDa and an isoelectric point of 4.55. The deduced amino acid sequence of REIC possesses a signal peptide (amino acids 1–22) and four potential *N*-glycosylation sites (at amino acids 96–99, 106–109, 121–124 and 204–207, respectively), suggesting that this protein is secreted after posttranslational modification. Furthermore, both the amino-terminus (amino acids 41–90) and carboxyl-terminus (amino acids 306–338) of this protein can form an extensive α-helix which is predicted to adopt a coiled-coil tertiary structure, suggesting that their functions involve protein-protein interaction. In addition, we found that its carboxyl terminus (amino acids 292–350) is rich in acidic amino acids (31.0%), such as aspartic acid (6.9%) and glutamic acid (24.1%). A search of the DDBJ database showed that the deduced amino acid sequence of REIC is identical to that of Dkk-3 protein, which is a novel secreted protein required for head induction in amphibian embryos and a potent Wnt inhibitor [19].

DISCUSSION

In this report we described the identification and characterization of the human REIC cDNA, which was expressed in normal human cells and down-regulated in human immortalized cell lines. The expression of REIC mRNA was also markedly reduced in eight of the nine tumor-derived cell lines examined. Only one cell line, T24, expressed it. Thus, it remains to be determined whether a point mutation or small deletion is present in T24 cells. The amino acid sequence of the REIC gene product was consistent with that of human *Dkk-3*, one of the Dkk family members. Recently, the expression of SK/Dkk-1, another member of the Dkk family, was also shown to be down-regulated in the human SK-OV-3 ovarian cancer cell line and the PA-1 human ovarian teratocarcinoma cell line [20]. Thus, down-regulation of Dkk family members may be involved in immortalization and cancer development of human cells. In fact, a transient expression of exogenous REIC significantly reduced the DNA-synthesis of the human immortalized KMST-6 cell line (data not shown). These findings indicate that the REIC protein may function as a tumor suppressor.

Regarding the Dkk gene family, Glinka *et al.* (1999) showed that *Xenopus* Dkk-1 inhibits Wnt-8 activities in *Xenopus* embryos and may play a role in induction of amphibian head structures [21]. Microinjection of Dkk-4 mRNA with Wnt-8 mRNA into *Xenopus* eggs also inhibited Wnt-induced axis duplication [19]. Similarly, co-expression of human Dkk-1 with Wnt-2 in NIH3T3 cells caused reversion of Wnt-2-induced morphological alterations [20]. Thus, some of the Dkk family members are likely to inhibit the Wnt-mediated signaling pathway.

There is evidence that activation of Wnt signaling contributes to the neoplastic process. Enhanced expression of Wnt caused a mammary tumor formation in mice [22, 23]. In cell cultures, several Wnt family members induced morphological alteration and increased saturation density of certain epithelial and fibroblast cells [24–27]. Similarly, genetic alterations of adenomatous polyposis coli (APC) and β-catenin, negative

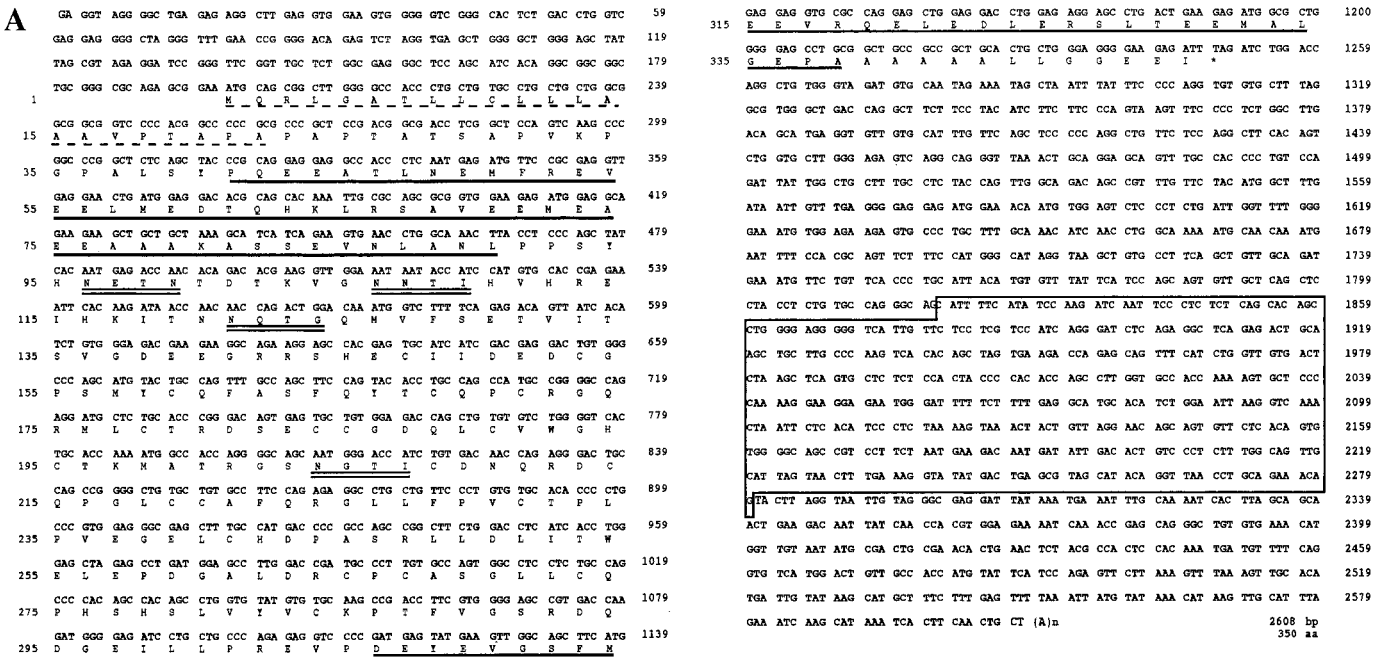

FIG. 4. (A) A full-length cDNA of the *REIC* gene and its deduced amino acid sequence. One-letter code is used for amino acids. Numbers on the right and left represent the nucleotide sequence and amino acid sequence, respectively. The coiled-coil tertiary structures are underlined. The signal peptide is dashed-underlined. The potential site of *N*-glycosylation is double-underlined. The cDNA fragment of the *REIC* gene isolated by the RDA system is boxed. (B) Schematic representation of the human REIC protein. CC, coiled-coil tertiary structures; SP, signal peptide; N, putative *N*-glycosylation site.

and positive regulators in the Wnt signaling pathway, respectively, have been observed in human colon cancer [28], melanomas [29], and hepatocellular carcinomas [30]. Overexpression of APC blocks cell cycle progression and causes apoptosis [31, 32]. Thus, aberrations of Wnt signaling pathways may be related to the development of some human cancers. Moreover, *c-myc* [32] and *cyclin D1* [33] oncogenes have been identified as targets of the Wnt pathway in colon cancer. Taken together, REIC, which is an inhibitor of the Wnt family, may function as a tumor suppressor gene.

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