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Mapping and Determination of the cDNA Sequence of the *Erc* Gene Preferentially Expressed in Renal Cell Carcinoma in the *Tsc2* Gene Mutant (Eker) Rat Model

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The Eker rat develops hereditary renal carcinomas (RCs) due to two hit mutations of the tumor suppressor gene, *Tsc2*. We previously identified using representational difference analysis (RDA), four genes that were expressed more abundantly in an Eker rat RC cell line than in normal kidney tissue. One gene, *Erc* (expressed in renal carcinoma) showed sequence homology to the mouse and human megakaryocyte potentiating factor (*MPF*)/*mesothelin* gene. The present study determines the full sequence of the cDNA and the exon–intron structure of the rat *Erc* gene and maps its locus in the chromosome by fluorescence *in situ* hybridization. Rat *Erc* and its human homologue were localized in chromosomes 10q12–21 and 16p13.3, respectively, both of which coincided with the locus of the *Tsc2/TSC* gene. We also found that *Erc* was expressed at higher levels in primary RCs compared with the normal kidney of the Eker rat. *Erc* may be related to carcinogenesis in the *Tsc2* gene mutant (Eker) rat model.

Key Words: **Eker rat; *Tsc2*; renal carcinomas.**

Several oncogenes and tumor suppressor genes have been identified by molecular genetic analysis of renal carcinomas (RCs). Four types of human hereditary RCs are presently known. These include von Hippel-Lindau (*VHL*) disease, hereditary papillary renal carcinoma, familial renal cancers with translocation of chromosome 3, and tuberous sclerosis. Originally reported by Eker in 1954, hereditary RC in the rat is an example of a dominantly inherited Mendelian predisposition to a specific cancer in an experimental animal (1, 2). A 1993 study showed that the causative gene for the Eker rat RC is linked to the proximal part of rat chromosome 10q (3, 4) where the synthetic gene arrangement corresponding to human 16p13.3 is conserved (5). Thereafter, the Eker rat was proved to have a germline mutation of the *Tsc2* gene, which is a rat homologue of the human tuberous sclerosis (*TSC2*) gene (6, 7) located in 16p13.3 in humans. Another somatic mutation of the *Tsc2* gene was later detected in the Eker rat RC (8–11), and furthermore, transduction of a *Tsc2* transgene suppressed renal cancer of the Eker rat (12, 13). Thus, the *Tsc2* gene was confirmed as a tumor-suppressor. Although the initial event that triggers Eker rat renal cancer is a somatic mutation of the *Tsc2* wild-type allele, other genetic or epigenetic modifications may also contribute to tumor progression. To search for such alterations, we identified genes that were expressed more abundantly in an Eker rat RC cell line than in the normal kidney by representational difference analysis (14, 15). We found the gene for the third component of complement (*C3*), the fos-related antigen 1 (*fra-1*) gene, the calpactine I heavy-chain (*annexin II*) gene, and the *Erc* (expressed in renal carcinoma) gene (15). After we determined the complete primary structure of rat *Erc* cDNA, we showed that the putative rat Erc product has 56.1% identity to human MPF/mesothelin. Rat *Erc* and its human homologue were localized by fluorescence *in situ* hybridization (FISH) in chromosomes 10q12–21 and 16p13.3, respectively, where the *Tsc2/TSC2* gene is also located. The level of *Erc* expression in the primary RCs was higher than that in the normal kidney of the Eker rat.

Sequence data have been deposited with GenBank Accession No. D87351.

Abbreviations used: RC, renal carcinoma; *Tsc2*, tuberous sclerosis 2; *Erc*, expressed in renal carcinoma; *MPF*, megakaryocyte potentiating factor; RDA, representational difference analysis.

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MATERIALS AND METHODS

Tissue samples and cell lines. Organs obtained from female Fisher rats and Eker rats were immediately frozen at −80°C until use. The rat RC cell lines LK9dL, LK9dR, and ERC cells including ERC33 (a gift from A. G. Knudson, Fox Chase Cancer Center) were established from primary renal tumors of Eker rats (6, 15). Other rat cell lines BP13 and BP36B, both of which are derived from a chemically (*N*-ethyl-*N*-hydroxyethylnitrosamine)-induced RC, were obtained from Dr. Tsuda (National Cancer Center Research Institute) (16). Human cell line O-3 was established from human renal cell carcinoma in our laboratory (manuscript in preparation). Other human cell lines, HeLa (cervical carcinoma), MCF-7 (mammary carcinoma), and HepG2 (hepatoblastoma) were obtained from American Type Culture Collection.

Complementary DNA cloning and sequence analysis. The *Erc* cDNA clone 15-2d1 was originally isolated by modified representational difference analysis (RDA) (15). The nucleotide sequence of 15-2d1 was determined using a T7 sequencing kit (Pharmacia) with [^{32}P]dCTP. The GenBank database was searched for homology.

Isolation and characterization of genomic clones. A Wistar rat genomic cosmid library was screened with a full length *Erc* cDNA as a probe and one clone (c1-1-1) covering the 3′-region of *Erc* gene was isolated. To isolate the 5′-region of the *Erc* gene, rat genomic DNA was Southern blotted with various restriction enzymes using a 5′-0.65 kb fragment of *Erc* cDNA as a probe. A single 3.5 kb band was detected by *Eco*RI digestion. Approximately 100 μg of rat (Brown Norway) genomic DNA was digested by *Eco*RI and separated by preparative 1% agarose gel electrophoresis. Fragments of DNA of around 3.5 kb were recovered from the gel and cloned into the *Eco*RI site of *Zap*II (STRATAGENE) to construct a sub-genomic library using the 5′-0.65 kb cDNA probe. Positive cosmid and phage clones were analyzed by restriction enzyme digestion followed by Southern hybridization with *Erc* probes. Sequences of exon–intron boundaries were determined using a cycle sequencing kit (Biosystems).

Rat genomic mapping. The 15-2d1 cDNA probe (15) was labeled with biotin-16-dUTP (Boehringer Mannheim) as described (17) and used for fluorescence *in situ* hybridization (FISH). Rat chromosomes prepared from early passages of cultured skin cells were analyzed using modified FISH and signal amplification (18–20). Slides stained with both 4′,6′-diamino-2-phenylindole (DAPI) and propidium iodide (PI) were observed under a Nikon OPTIPHOT-2-EFD2 microscope (B-2A filter for PI staining, UV-2A filter for DAPI staining) and photographed using Fuji chrome film (Sensia, ASA 100).

Human genomic mapping. R-banded chromosomes were prepared by standard means (21) with some modifications (22). Two human *MPF* genomic clones (pHGKPOA and pHGKPOC) used as probes for FISH were supplied by Chugai Pharmaceutical Co., Ltd. (unpublished). The probes were labeled with biotin-16-dUTP (Boehringer Mannheim) by nick translation and hybridized as described (23). Background signals were reduced by competition with whole human genomic DNA (added to the hybridization solution). Signals were detected with fluorescein-avidin DCS (Vector Laboratories). The slides were stained with propidium iodide (0.5 μg/ml; Sigma) and examined under a Nikon Optiphot-2-EFD2 microscope (B2A filter for R-banded chromosomes and UV-2A filter for G-banded chromosomes), then chromosomes were microphotographed using Fuji (Sensia, ASA100) chrome film.

Partial hepatectomy. Two-thirds partial hepatectomy in the Fisher rat was performed by ligation and excision of the median and left lobes close to the hilus of the liver. Then at 20 h after surgery, the rat was sacrificed and RNA was obtained from the liver.

RNA isolation and Northern blots. RNAs were isolated by acid guanidinium thiocyanate-phenol-chloroform extraction using Isogen (Nippon Gene). RNA samples (10 μg) were prepared for Northern blotting by separation on 1% agarose gels in 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer containing 16.6% formaldehyde and transfer onto nylon membranes (Biodyne B, Pall). Pre-hybridization and hybridization with ^{32}P-labeled probes proceeded in 0.2 M phosphate buffer (pH 7.2) containing 1 mM EDTA, 1% BSA and 7% SDS at 65°C. The membranes were washed twice in 1× SSC (0.15 M NaCl, 15 mM sodium citrate), 0.1% SDS for 15 min at room temperature, once in 1× SSC, 0.1% SDS for 30 min at 65°C and exposed to X-ray film (Kodak).

RESULTS

Primary Structure of the Erc cDNA

The cDNA sequence of clone 15-2d1 was 2117 bp long (Accession No. D87351) and contained a 1926 bp open reading frame (ORF) that was proceeded by an in frame termination codon (Fig. 1). A potential signal sequence for polyadenylation (AATAAA) was located immediately upstream of the 3′poly(A) tail. The first ATG (nt. 91–93) of this ORF was embedded in the Kozak's consensus sequence to produce a methionine residue. Considering this first methionine as an initiator, the predicted amino acid sequence encoded by this cDNA consisted of 625 amino acid residues. A database search revealed that this predicted amino acid sequence had 87.4 and 56.1% identity, respectively, to mouse and human megakaryocyte potentiating factor (*MPF*)/*mesothelin* (Fig. 2). At the nucleotide sequence level, *Erc* showed 90.6 and 67.6% identity with mouse and human *MPF/mesothelin* cDNA, respectively. Two hydrophobic regions, a putative signal peptide near the amino-terminus and a putative glycosylphosphatidylinositol anchorage sequence near the carboxy-terminus, have been identified in human *MPF/mesothelin* (24). These hydrophobic regions were conserved in *Erc* as well as in the mouse homologue. A putative furin cleavage sequence was also conserved in rat *Erc*, although one arginine was substituted by histidine (25). Three potential N-linked glycosylation sites were identified in rat Erc instead of the four sites in the human product (Fig. 1). Two types of cDNA are derived from the human *MPF/mesothelin* gene by differential splicing (25). Figure 2 shows the longer type of human *MPF/mesothelin* (amino acids 409–411 are missing in the shorter type) and that the Erc product encoded by the 15-2d1 corresponded to the shorter type.

Exon–Intron Structure of the Rat Erc Gene

We determined the structure of rat *Erc* gene using cosmid (c1-1-1) and phage (1A-1-1) clones that covered the 3′- and 5′-genomic regions, respectively, from the *Eco*RI site in exon 7 of the *Erc* gene. Sequence analysis revealed that the rat *Erc* gene consists of 16 exons spanning approximately 5.4 kb. All of exon–intron junctions followed the GT/AG rule for splicing donor/acceptor sites (Fig. 3). Because the cDNA and genomic DNAs were isolated from several different rat strains, polymorphic sequence differences were found between

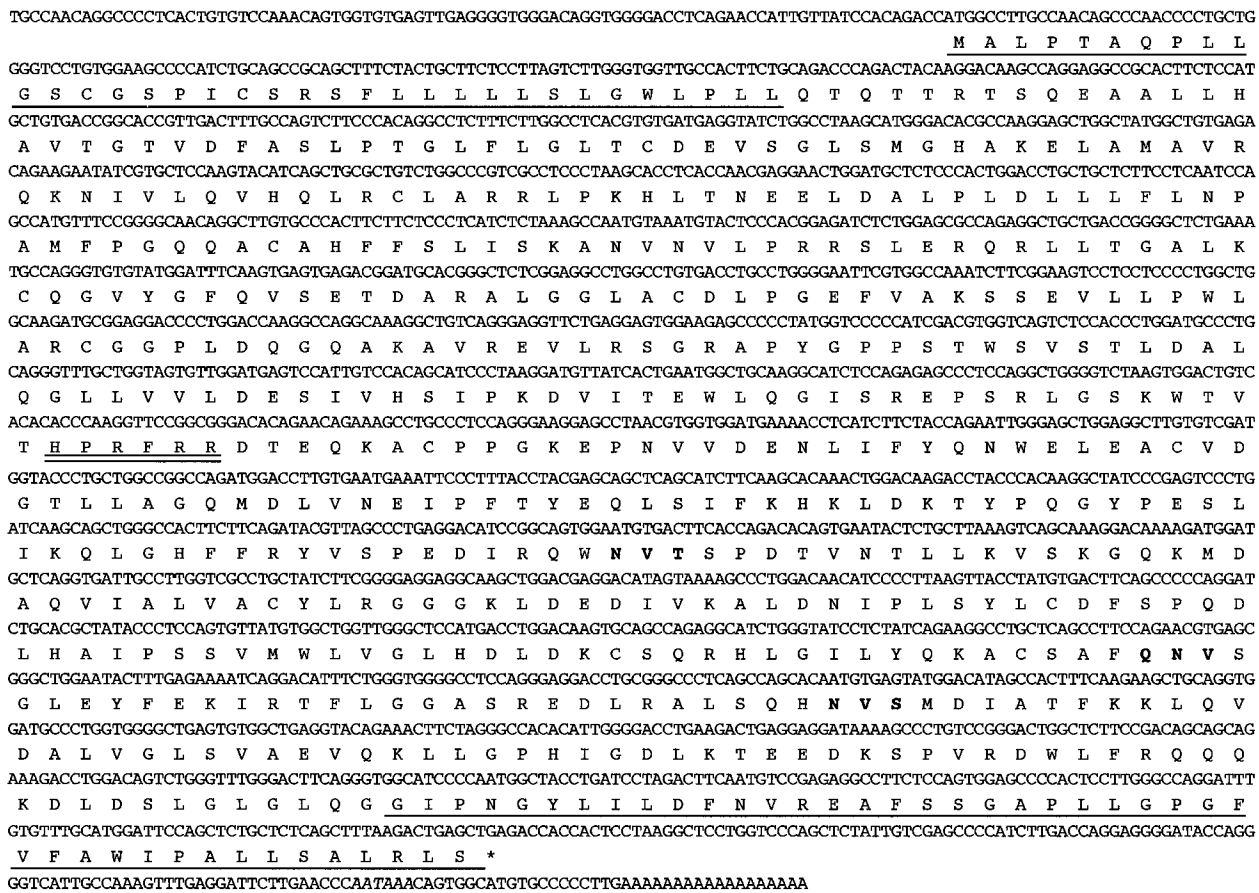

FIG. 1. Nucleotide and deduced amino acid sequence of *Erc* cDNA. Nucleotide sequence (upper line) and deduced amino acid sequence (lower line) of the *Erc* cDNA which is 2117 bp long and contains an open reading frame (ORF) of 1926 bp preceed by an in frame termination codon. The first ATG of this ORF is nt. 91–93. Star indicates stop codon. Putative signal peptide near amino-terminus and putative glycosylphosphatidylinositol anchorage sequence near carboxy-terminus are underlined. Putative furin cleavage sequence is double underlined. Three potential N-linked glycosylation sites are noted in boldface. Polyadenylylation signal (AATAAA) is located 20 bp upstream from the poly(A) tail.

the cDNA and genomic DNA (data not shown). The overall exon–intron structure of rat *Erc* was identical to those of the human and mouse counterparts (data not shown).

Chromosome Mapping of Rat Erc and Human MPF/Mesothelin

We analyzed the rat chromosome by FISH using 15-2d1 cDNA as a probe to determine the chromosomal localization of the *Erc* gene. Among 50 rat (pro)metaphase cells hybridized with the probe and observed under the microscope, 35 cells had symmetrical double spots on chromosome 10 at band q12–q21 (Figs. 4A and 4B). No other chromosomes exhibited double spot signals. We therefore concluded that the rat *Erc* gene is located in rat chromosomal region 10q12–q21. For comparison, we also analyzed a human chromosome by FISH using two human *MPF/mesothelin* genomic clones as probes. Fifteen of 50 (pro)metaphases examined showed symmetrical double spots on at least one homologue of human chromosome 16. The signal region was localized to 16p13.3 (Figs. 4C and 4D). No other chromosomes exhibited double spot signals. We therefore concluded that the human *MPF* gene is located in chromosomal region 16p13.3.

Erc mRNA Expression in Rat and Human Tissues and Various Cancer Cell Lines

We examined the expression of *Erc* mRNA in the brain, heart, lung, liver, spleen, kidney, ovary, and skeletal muscle of five normal adult female Fisher rats by Northern blotting. *Erc* mRNA (2.4 kb) was expressed in the rat lung as it was in humans. However, the level of *Erc* expression was below the limit of detection in all other tissues examined (Fig. 5). In seven of ten adult female Eker carriers, we detected high levels of *Erc* mRNA expression in primary RCs as well as in the lung (Fig. 5). Expression of *MPF*, human

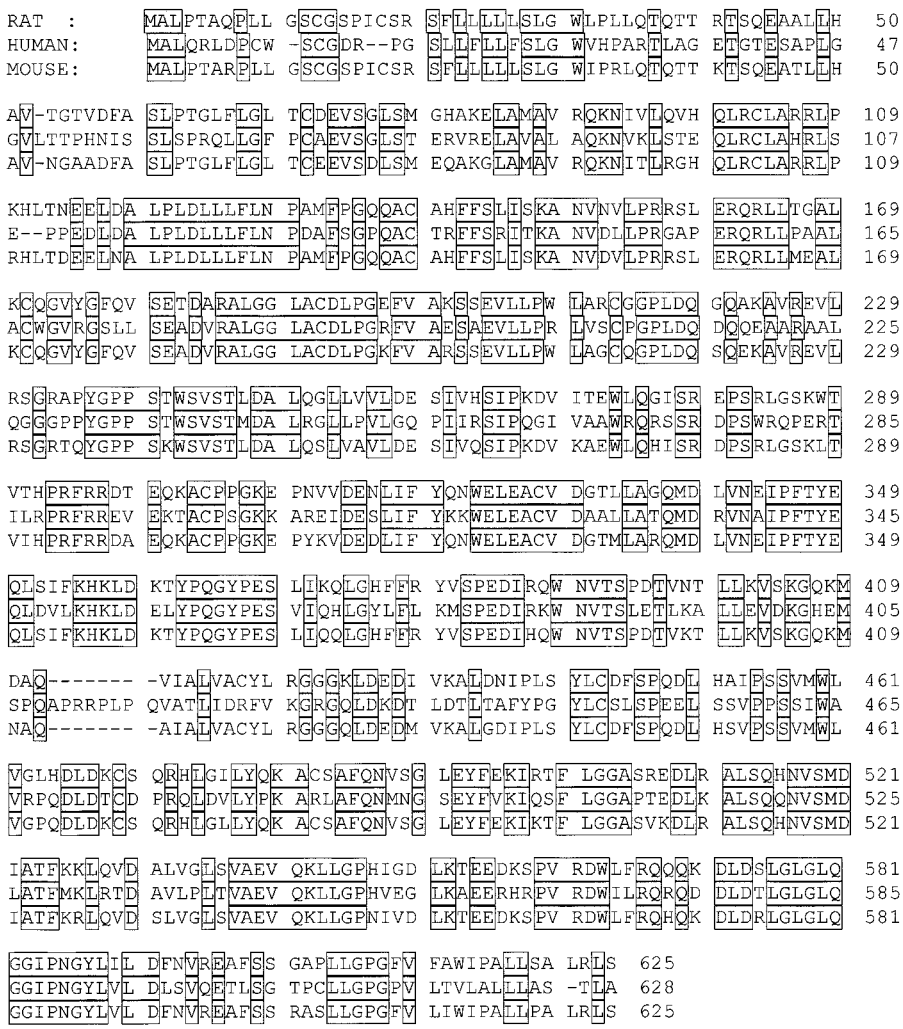

FIG. 2. Comparison of predicted amino acid sequences of rat Erc as well as human and mouse MPF/Mesothelin gene products. Boxed areas show homology.

homologue of *Erc*, was detected in human cell lines HeLa (cervical carcinoma) and O-3 (renal cell carcinoma), but not in MCF-7 (mammary carcinoma), HepG2 (hepatoblastoma) (Fig. 6). *Erc* was not expressed in the chemically-induced rat RC cell lines and the rat liver before and after partial hepatectomy (Fig. 6).

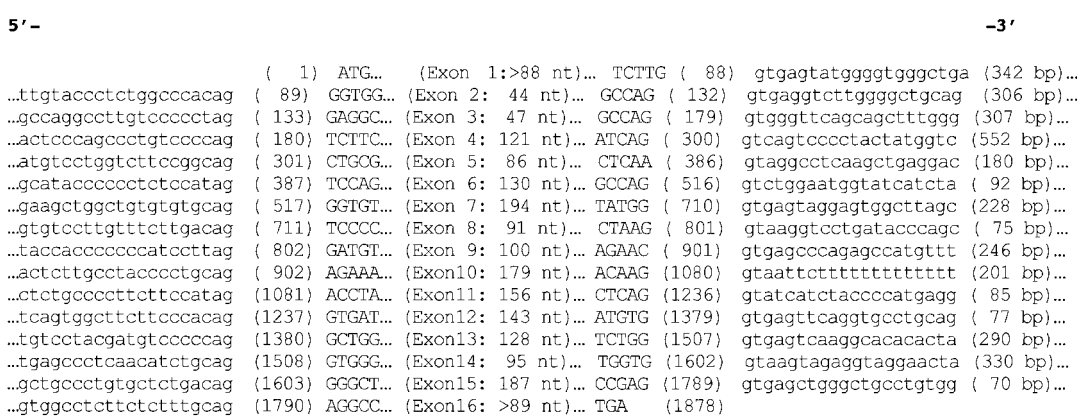

FIG. 3. Exon–intron boundaries of rat *Erc* gene. Nucleotide sequences of exon–intron boundaries and size of intron and exon are shown.

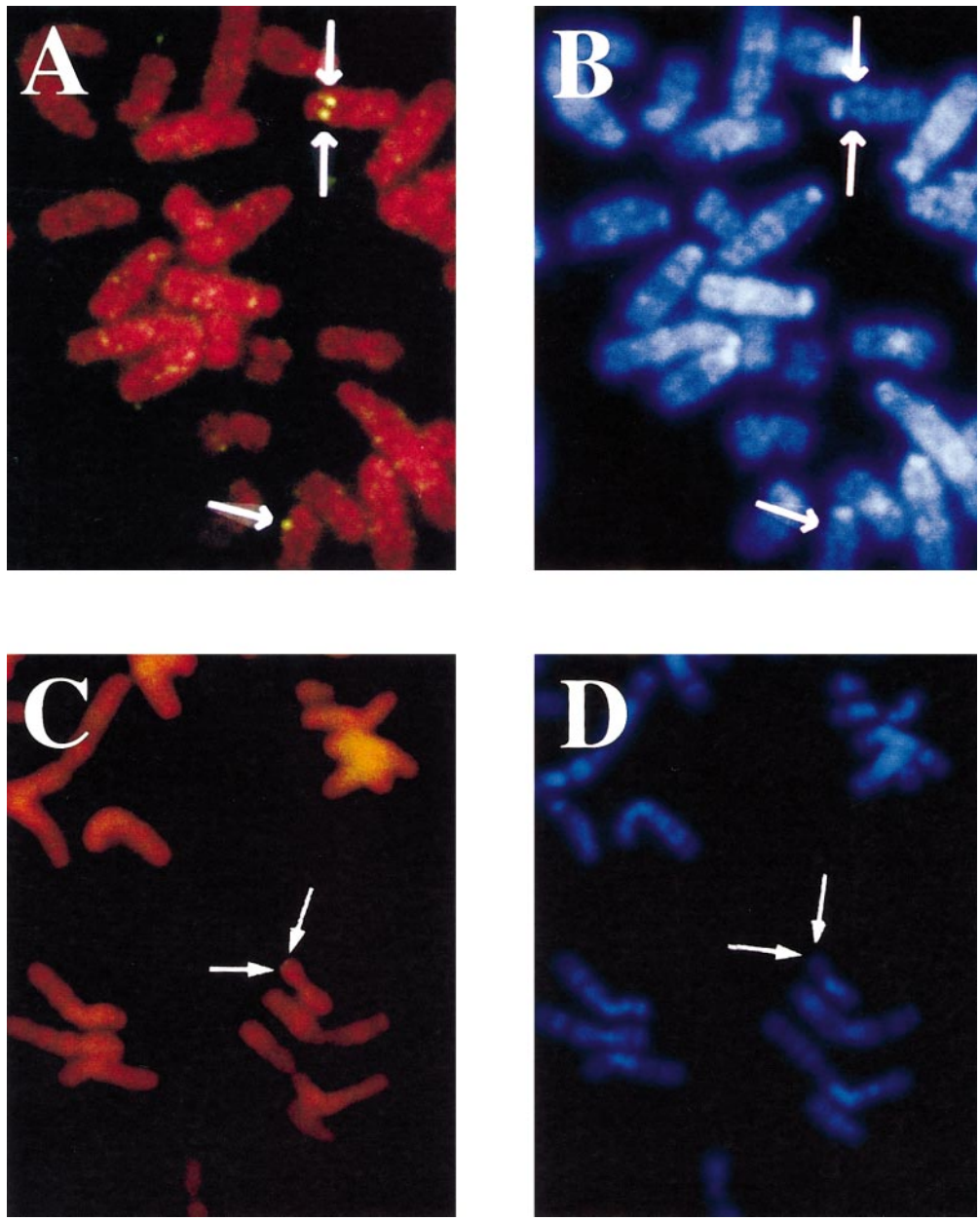

FIG. 4. Chromosome localization of rat *Erc* and its human homologue. (A and B) Rat partial metaphase chromosomes after FISH using rat *Erc* cDNA probe. (A) PI-stained chromosomes. Arrows indicate symmetrical double spots of TG8 copies. (B) Same chromosomes stained with DAPI (UV-2A filter). Observations using both staining filters located these symmetrical double spots on chromosome 10q12–q21. (C and D) Human *MPF* localized to human chromosome 16p13.3 by FISH with (C) R-banded chromosomes. (D) Same chromosomes with G-like bands. Arrows: Signal spots after FISH using biotinylated human *MPF* genome DNA.

DISCUSSION

We isolated subtracted cDNA clones that expressed high levels of *Erc* in Eker renal carcinoma cells, then used a modified RDA method to search for genes specifically involved in renal carcinogenesis (15). This procedure identified the third component of the complement (*C3*) gene, the fos-related antigen 1 (*fra-1*) gene, the calpactine I heavy-chain (*annexin II*) gene, and the *Erc* gene. A comparison of gene expression profiles between tumors and corresponding normal tissues is one approach to understanding the molecular events associated with neoplastic transformation. The *Erc* gene is barely detectable in the normal rat kidney and thus may be a candidate for involvement in renal carcinogenesis. Originally, *Erc* was characterized as a novel gene because its partial cDNA sequence showed no significant homology with other known sequences according to a database search (15).

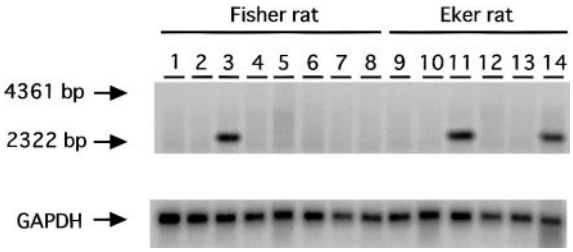

FIG. 5. *Erc* expression in rat tissues identified by Northern blotting. Total RNAs derived from Fisher rat (lanes 1–8) and Eker rat (lanes 9–14). Lanes: 1, brain; 2, heart; 3, lung; 4, liver; 5, spleen; 6, kidney; 7, ovary; 8, skeletal muscle; 9, brain; 10, heart; 11, lung; 12, liver; 13, kidney; 14, renal carcinoma. Labeled 15-2d-1 was the probe. In addition to the lung, primary RCs from seven out of ten adult female Eker carriers expressed high levels of *Erc* mRNA.

We determined the complete primary structure of rat *Erc* cDNA in the present study, then found high homology between *Erc* and human or mouse cDNAs encoding *MPF/mesothelin* (24, 26). *Mesothelin* was originally identified as a cell surface antigen that is recognized by the monoclonal antibody K1 in human mesotheliomas and ovarian cancers (27, 28). *MPF* was independently identified as a soluble factor from the supernatant of cultured human HPC-Y5 pancreatic cancer cells, which stimulates the megakaryocyte colony forming activity of IL-3 *in vitro* (29). However, cDNA cloning revealed that mesothelin and MPF are derived from a common ~70-kDa precursor by protease cleavage (24, 27). The ~30-kDa amino-terminal half (MPF) is related to the extracellular region and the ~40-kDa carboxy-terminal half (mesothelin) remains attached to the cell surface by a GPI-anchor (27). Two hydrophobic regions, an amino-terminal signal peptide and a carboxy-terminal hydrophobic region that may act as a signal for the attachment of GPI-anchor, and a putative furin cleavage site are thought to be involved in this maturation process (30, 31). All of these structural characteristics were conserved in the rat Erc product, suggesting a similar maturation process upon Erc protein expression. Homology between Erc and mesothelin/MPF also extended to the entire molecule. Many cysteine residues were conserved. These residues may be involved in the formation of the higher order protein structure seen in some cell surface receptor families via disulfide bond formation (32). In addition, the rat *Erc* gene consists of 16 exons and has polymorphic sequence differences (33). The *Erc* gene was localized on rat chromosome 10q12–21 near the *Tsc2* gene. We also determined the chromosomal localization of the human *MPF/mesothelin* gene on chromosome 16p13.3. A syntenic relationship between rat chromosome 10q12–21 and human chromosome 16p13.12-13.3 has been demonstrated (4, 5, 34). The chromosomal localization in both species together with the structural conservation suggest that rat *Erc* and human *MPF/mesothelin* are functional orthologues. High levels of *MPF/mesothelin* mRNA are expressed in the human lung (24). Similarly, the level of rat *Erc* mRNA expression was highest in the lung among normal tissues examined here, again suggesting functional conservation between *Erc* and *MPF/mesothelin*. However, the cell types expressing *Erc* in the lung as well as in other organs and tissues remain to be precisely determined. Our earlier effort to identify *Erc* was performed using renal carcinoma cell lines (15). An examination of primary renal carcinomas from Eker rats in the present study showed higher expression of *Erc* than in the normal kidney. This suggests that *Erc* is expressed at high levels in renal carcinoma *in vivo*.

Detailed histological analysis using mRNA hybridization *in situ* or an anti-Erc antibody should define the cell-type specificity of *Erc* expression in normal rat tissues and during renal carcinogenesis. The physiological function(s) of the *Erc* product has not yet been elucidated. However, some information about the function of human *MPF/mesothelin* has been reported (35). *Mesothelin* is thought to be involved in invasion as it is expressed at high levels in some tumors showing aggressive peritoneal spreading and/or local invasion such as malignant mesotheliomas and ovarian carcinomas (25). As reported previously, *MPF/mesothelin* was not similarly expressed in human cancer cell lines (25). Interestingly, chemically (*N*-ethyl-*N*-hydroxyethylnitrosamine)-induced non-Eker rat renal carcinoma cell lines did not express *Erc* (Fig. 6). Moreover, *in vivo* examination using partial hepatectomy did not support the idea that the expression of *Erc* was merely upon cell proliferation (Fig. 6). It is notable that there was the difference between spontaneous (human and Eker rat) and chemically-induced non-Eker rat renal carcinomas, although we do not have any good explanation for this difference at present. As *mesothelin* is a cell

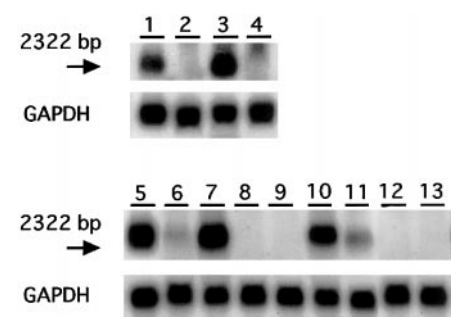

FIG. 6. *MPF, Erc* human homologue, RNA levels in cell lines and *Erc* expression in rat cell lines, normal liver and regenerative liver after partial hepatectomy. Samples (10 μg) of total RNA from O-3 cells (human RCC cells: lane 1), MCF-7 cells (lane 2), HeLa cells (lane 3), HepG2 cells (lane 4), rat lung (positive control: lane 5), LK9dR (lane 6), ERC33 (lane 7), BP13 (chemically-induced RC: lane 8), BP36B (chemically-induced RC: lane 9), ERC18 (lane 10), ERC19 (lane 11), normal rat liver (lane 12), regenerative rat liver after partial hepatectomy (lane 13). Labeled *MPF* was used as the probe of human (lanes 1–4). Labeled *Erc* was used as the probe of rat (lanes 5–13).

surface protein, it may function as a cell-adhesion molecule or as a cell surface receptor for some ligands (36, 37). Our preliminary transfection data also suggest a role of the Erc product in cell adhesion and/or cell shape dynamics (Yamashita *et al.*, unpublished observation). Whether or not processing of the *Erc* precursor is related to the extracellular region and whether or not *Erc* acts as a megakaryocyte potentiating factor, cell-adhesive, or invasive factor of cancer remain to be elucidated. The amino-terminal portion of Erc, if it is involved, may have another biological effect(s). Thus the Tsc2 mutant (Eker) rat provides a promising model for analyzing the essential events of carcinogenesis at different stages. In addition, we found the *MPF, Erc* human homologue, expression in the human renal cell carcinoma cell lines. It shows the potential of *Erc* as a tumor marker for renal cell carcinoma. All of these notions regarding *Erc* function(s) and its association with tumor formation should be further investigated.

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