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Overexpression of Members of the AP-1 Transcriptional Factor Family from an Early Stage of Renal Carcinogenesis and Inhibition of Cell Growth by AP-1 Gene Antisense Oligonucleotides in the *Tsc2* Gene Mutant (Eker) Rat Model

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We previously isolated subtracted cDNA clones for genes having increased expression in *Tsc2* gene mutant (Eker) rat renal carcinomas (RCs). Among them, *fra-1* encoding a transcriptional factor activator protein 1 (AP-1) was identified. We have therefore investigated whether other members of the AP-1 transcription factor family might also be involved in renal carcinogenesis in the Eker rat model. In the present study, overexpression of *fra-1, fra-2, c-jun, junB,* and *junD* mRNAs was demonstrated in RCs by Northern blot analysis. Interestingly, AP-1 proteins were highly expressed even in the earliest preneoplastic lesions (e.g., phenotypically altered tubules) as suggested by immunohistochemistry. Moreover, 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (TRE)-binding activity of AP-1 proteins was observed in RC cell extracts by electrophoretic mobility shift assay. As a next step, we transfected antisense oligonucleotides targeting AP-1 genes into RC cells and demonstrated that their growth was strongly inhibited. Thus, the data suggest that overexpression of AP-1 genes might play a crucial role in renal carcinogenesis in the Eker rat model. © 1997 Academic Press

The hereditary renal carcinoma (RC) in the rat, originally reported by R. Eker in 1954, is an example of a dominantly inherited Mendelian predisposition to a specific cancer in an experimental animal (1). Recently, we and others have reported that a germline insertion in the rat homologue of *TSC2* gene gives rise to the dominantly inherited cancer in the Eker rat model (2, 3). We previously found that a second somatic mutation (second hit) might be a rate-limiting step for renal carcinogenesis in the Eker rat model, as well as providing evidence for a tumor suppressor nature of *Tsc2* (4-7). Finally, we constructed transgenic Eker rats with a wild-type *Tsc2* gene and ascertained that germline suppression of the Eker phenotype is possible for both embryonic lethality in homozygotes and tumor development in heterozygotes, confirming that the predisposition for RCs in the Eker rat is caused by the *Tsc2* germline mutation (8). At the histological level, tumors in this model develop through multiple stages from early preneoplastic lesions (*e.g.*, phenotypically altered renal tubules) to adenomas in virtually all heterozygotes by the age of 1 yr (4), facilitating analysis of the essential events for carcinogenesis. Heterozygosity is not itself a sufficient condition for the development of cancer, but only one hit is enough to produce precursor lesions in the Eker rat (4, 5). These are initially benign, but continued proliferation virtually ensures that other critical, though not rate-limiting events, will occur (9).

We previously isolated four subtracted cDNA clones with increased expression in *Tsc2* gene mutant (Eker) rat RCs, being the *fra-1* gene encoding a transcriptional factor activator protein 1 (AP-1) identified among them (10). The AP-1 family is composed of *jun*, c-Jun, JunB and JunD, and *fos*, c-Fos, FosB, Fra-1, Fra-2 gene products. The induction of their gene expression does not require intervening protein synthesis, and thus these immediate early genes are thought to be the first genes activated by mitogenic stimuli. They are therefore considered to play key roles in signal transduction pathways involved in complex cellular growth, differentia-

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TABLE 1

fosB	a) 5′-GGATGTTGACCCTGGCAAATCTCTCACCTC-3′, b) 5′-TGTGTGTAAAGAGAGAAGCCGTCAGGTTGG-3′, c) 5′-AGGAAGTGTACGAAGGGCTAACAACGGGGA-3′,
fra2	a) 5′-CTGGGTCTTGTCCAGCCCTGCTGAGGAAGA-3′, b) 5′-CACTGGGGTAGGTGAAGACAAGGTTTGAAG-3′, c) 5′-GATGACTGGTCCCCACTGCTACTGCTTCTG-3′,
junB	a) 5′-TGTGTCTGATCCCTGACCCGAAAAGTAGCT-3′, b) 5′-AAAGGGTGGTGCATGTGGGAGGTAGCTGAT-3′, c) 5′-GGTTCTCAGCCTTGAGTGTCTTCACCTTGT-3′,
junD	a) 5′-CTGAGCGTCAGCGCGTCTTTCTTCAGCATG-3′, b) 5′-GTTGGACTGGATGATCAGCCTTTCCAGCTC-3′, c) 5′-ACCTTAGGGTAGAGGAACTGCGTACTGGTC-3′,

tion, stress, and tumorigenesis (11, 12). The proteins themselves locate in the nucleus and form homodimers or heterodimers through leucine zipper structure interactions. Jun family members can form low-affinity homodimers as well as high-affinity heterodimers with the Fos family members, whereas Fos-related proteins do not form stable homodimers. The resultant AP-1 complexes can function as transcription factors through binding to the DNA consensus sequence TGAc/gTCA (13), otherwise known as the AP-1 site or 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (TRE) that is contained within the promoter region of many genes active in cell proliferation, differentiation, and tumorigenesis (14).

Transgenic mice with stable expression of c-*fos* have been reported to demonstrate dysregulation of bone growth eventually resulting in osteosarcomas and chondrosarcomas (15). Others expressing an oncogenic form of *jun* develop fibrosarcomas at sites of wound healing (16). Furthermore, high-level expression of most members of the *fos* and *jun* gene families has been reported to cause cellular transformation of chicken embryonic fibroblasts (17, 18). Aberrant expression of AP-1 genes has been observed in several but not all malignant cells. The available data indicate an essential relevance to neoplastic transformation in specific tissues, but the situation with regard to expression of AP-1 genes in renal carcinogenesis has hitherto been unclear.

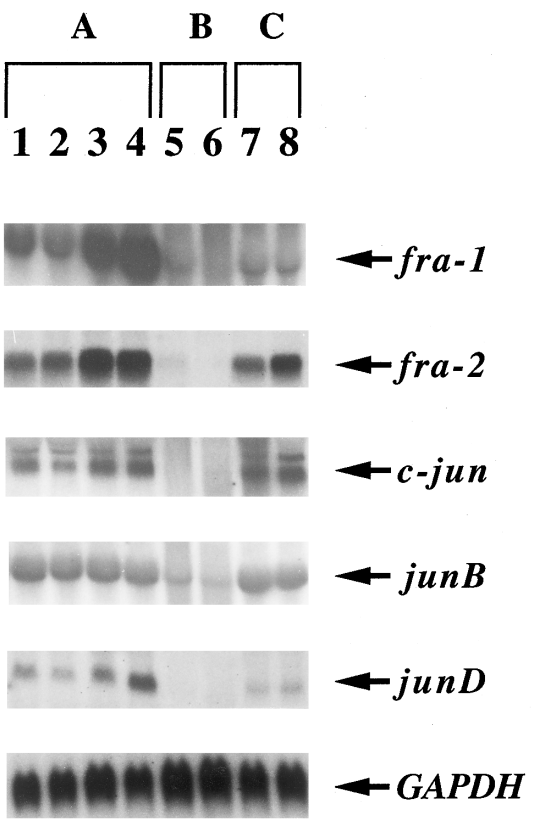
In this study, we therefore investigated expression of AP-1 mRNAs and proteins during renal carcinogenesis in the Eker rat model. As a next step, antisense oligonucleotides targeted against AP-1 genes were transfected into RC cells to assess the role of their overexpression in tumor development.

TABLE 2

c-fos	5′-ACCCGAGAACATCAT-3′
fosB	5′-AAAAGCTTGAAACAT-3′
fra-1	5′-GAAGTCTCGGTACAT-3′
fra-2	5′-ATAATCCTGGTACAT-3′
c-jun	5′-CATCTTTGCAGTCAT-3′
junB	5′-CATTTTCGTGCACAT-3′
junD	5′-GAAGGGCGTTTCCAT-3′

MATERIALS AND METHODS

Tissue samples and cell lines. Tissues were dissected from kidneys of adult male non-Eker/ Eker rats and primary renal tumors, and frozen at −80°C until use. LK9d (2) and ERC18, ERC27, ERC31



A: Renal carcinoma cell lines
B: Normal kidney tissues
C: Primary renal carcinoma tissues

FIG. 1. Northern blot analysis of *jun* and *fos* gene family member mRNAs. Total RNAs were isolated from four renal carcinoma (RC) cell lines (lane 1, ERC18; lane 2, ERC27; lane 3, ERC31; lane 4, LK9d), two normal kidneys from non-Eker rats (lanes 5 and 6) and two primary RC tissues from Eker rats (lanes 7 and 8). Northern blot analysis performed sequentially with ^{32}p-labeled cDNA probes, demonstrated overexpression of *fra-1, fra-2, c-jun, junB* and *junD* in all RC cell lines and tissues when compared with normal kidney tissues. mRNA for *c-fos* could not be detected, whereas a small mount of mRNA of *fosB* was found to be present in RC cell lines and tissues.

(gift of A. G. Knudson, Fox Chase Cancer Center) (4) cell lines were established from primary renal tumors of Eker rats. All cell lines were grown in medium as described earlier (4).

Northern blot analysis. Total RNAs were isolated from the four RC cell lines (LK9d, ERC18, ERC27 and ERC31), RC tissues from Eker and normal kidney tissues from non-Eker rats by extraction in a solution containing guanidium isothiocyanate and phenol. 20 μg aliquots were analysed by northern blot hybridization. RNAs were denatured, separated by electrophoresis in 1.5% formaldehyde agarose gels, and transferred onto nylon membranes (Biodyne B, Pall) by capillary blotting in 20 × SSC. Prehybridization was carried out in 0.2 M phosphate buffer (pH 7.2), 1mM EDTA, 1% bovine serum, 7% sodium dodecyl sulfate at 60°C or 65°C. After addition of denatured ^{32}P dCTP-labeled DNA probes prepared by random oligonucleotide-priming, hybridization was carried out in the same solution at 60°C or 65°C. As probes, we used cDNAs of human *c-fos, fra2, junB, junD,* and mouse *c-jun,* kindly provided by Dr. M. Fujii (Dept. of Immunotherapeutics, Tokyo Med. Dent. Univ.), and rat *fra-1* cDNA. Furthermore, as the human cDNAs used in this study may fail to hybridize rat mRNAs, a mixture of the 3 oligonucleotides a-c, end-labeled via T4 polynucleotide kinase with ^{32}P dATP was also used as a probe (Table 1). These oligonucleotides were selected since they could not hybridize with RNA sequences of the other AP-1 family members each others. The filters were washed twice in 1 × SSC (0.15 M NaCl, 15 mM sodium citrate), 0.1% SDS for 15 min at room temperature and washed once in the same solution for 15 min at 65°C before autoradiography.

Gel mobility-shift analysis. In order to detect DNA-binding of transcription factor AP-1, we used as a probe for the assay a 17bp synthetic double-stranded DNA oligonucleotide (GATCGTGACTCAGCGCG) containing the TRE consensus sequence derived from the methallothionein gene promoter (19). Nuclear extracts from renal carcinoma cell lines (LK9d) were prepared by the method described previously (20). Preincubation was carried out with 5 μg protein of nuclear extract and 2 μg of poly(dI:dC) in 20 μl of M buffer containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [pH7.9]), 40 mM KCL, 0.2 mM EDTA, 8 mM $MgCl_2$, 10% glycerol, 2% polyvinyl alcohol, and 1 mM DTT (dithiothreitol) for 5 min at room temperature. Approximately 1ng of [^{32}P]-labeled double strand synthetic oligonucleotide was added to the reaction mixture and incubation continued for an additional 20 min. The complex formed was separated by electrophoresis in a 4% polyacrylamide gel with 0.25 × Tris-borate-EDTA buffer containing 12.5 mM Tris-borate, pH8.3, 0.25 mM EDTA. The gels were dried and then authoradiographed with X-ray film.

Effects of AP-1 gene antisense oligonucleotides on LK9d cell number. In designing the antisense sequences for all AP-1 genes, we analyzed the specificity (the degree of cross-hybridization against the entire sequences in the GeneBank database) of each nucleotide, and the possibility of forming secondary structures in both oligonu-

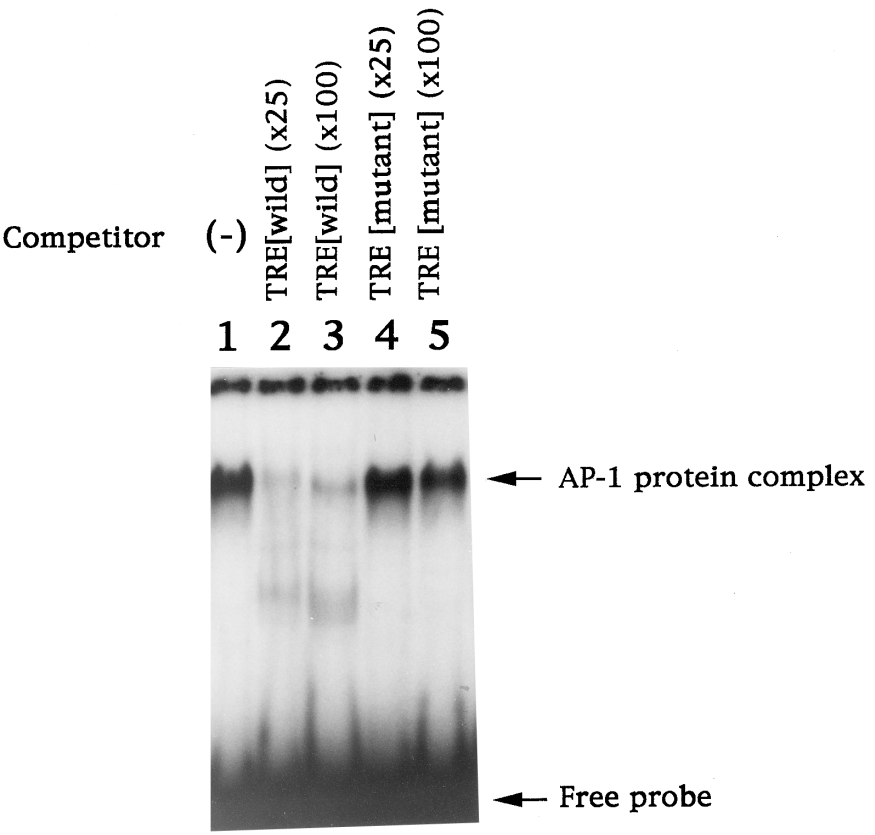


FIG. 2. Sequence specificity of AP-1-TRE binding activity in the LK9d renal carcinoma (RC) cell line as determined by electrophoretic gel mobility-shift assay. A nuclear extract was isolated from LK9d and a gel retardation assay was performed (lanes 1–5) using a ^{32}p-labeled DNA oligomer containing one AP-1 consensus sequence (TRE). The binding reaction was carried out in the presence of 25- and 100-fold excess of cold oligonucleotides as competitors [TRE; (lanes 2 and 3), respectively] [mutant TRE; (lanes 4 and 5), respectively]. While the free probe was found at the bottom of the gel, the AP-1 protein complex migrated more slowly. The competition demonstrated the specificity of the assay.

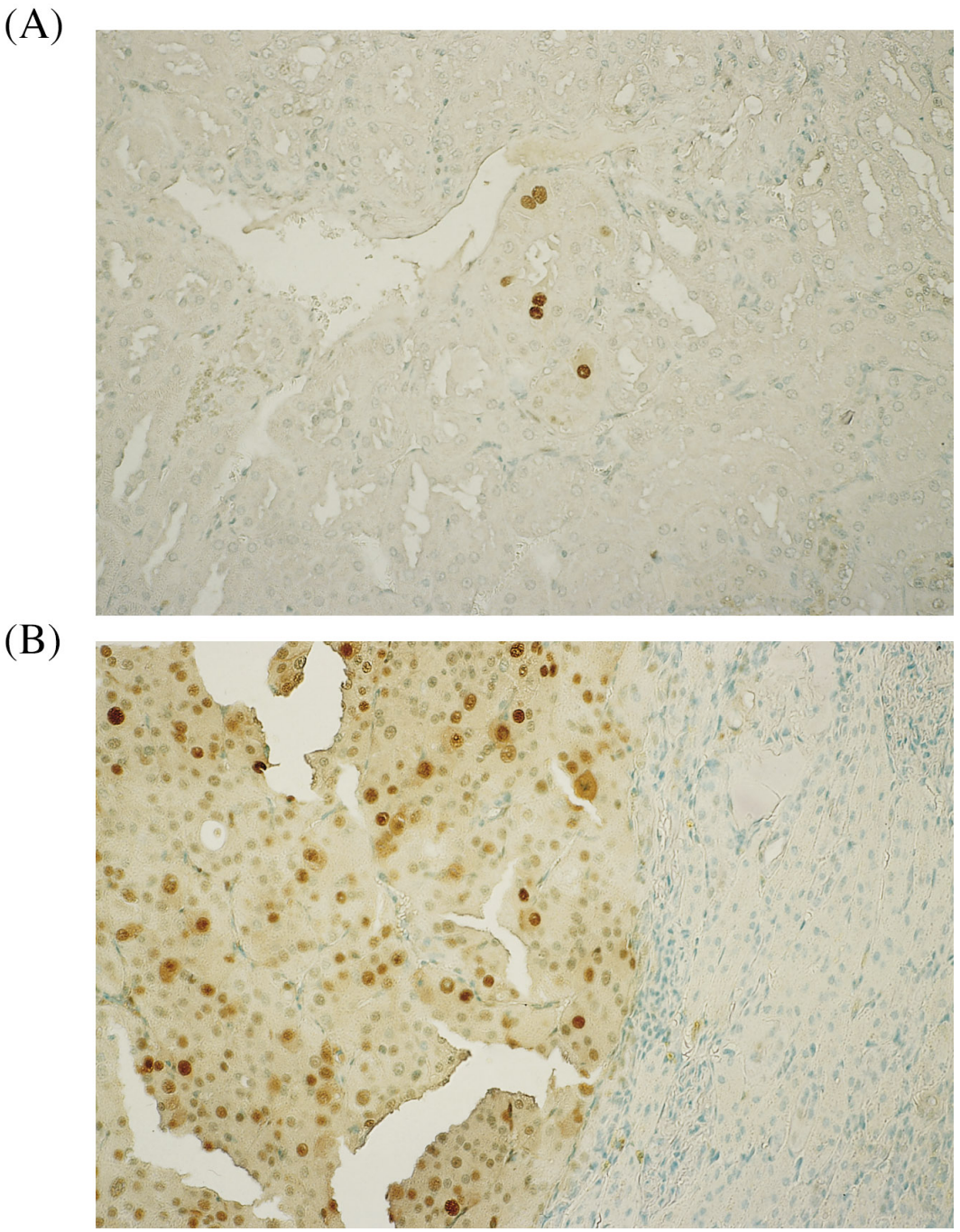


FIG. 3. Immunohistochemistry for c-Jun. (A) The earliest preneoplastic lesions (phenotypically altered tubules) (original magnification × 20). (B) Renal carcinomas (original magnification × 20).

cleotides and the target mRNA. Each antisense sequence was chosen as a 15-mer sequence containing the AUG translation initiation codon because this is short enough to penetrate living cells but long enough for specificity (21). They are shown in Table 2. As the control sense oligonucleotide, (5′-ATGACTGCAAAGATG-3′) was used. Phosphorothioate-modified oligonucleotides were synthesized. For the oligonucleotide treatment of LK9d cells, the cells were seeded at an initial concentration of 2×10^5 cells/ml, and washed twice with PBS(−) after two rounds of passage. After resuspension in 1000 μl of medium containing only 0.5% FBS to avoid degradation of oligonucleotides in the presence of high concentration serum, they were seeded onto 60 mm plates at the density of 3×10^4 cells/ml. Incubation was with or without a 5 μM concentration of antisense or control sense oligonucleotides for 12 h, followed by a second treatment after a 12 h interval. Serum was then added to give a final concentration of 5% and the cultures were continued for an additional 1-4 days. Cell numbers were counted daily, using a hemocytometer; Cells in four corner squares (total 64 small squares) were counted, and the

number of total cells was calculated. The person counting was blinded to the experimental condition. LK9d cells was also treated with a combination of all 7 kinds of antisense oligonucleotides against AP-1 genes each at 2.5 μM (total concentration, 17.5 μM).

Immunohistochemistry of AP-1 proteins. RCs, the earliest preneoplastic tissues (e.g., phenotypically altered renal tubules) and normal kidney tissues adjacent to the tumors were immunohistochemically analyzed. The tissue samples were fixed in 10% buffered formalin (pH7.0) for 24 hr immediately after sacrifice, and routinely processed for embedding in paraffin wax. The serial sections were cut at 5 μm thickness and placed onto poly-L-lysine coated glass slides, dewaxed and rehydrated for AP-1 immunostaining and haematoxylin and eosin (H&E) staining for histological evaluation. For the former, the slides were microwaved at 700 W in 0.1 M citrate buffer (pH6.0) twice for 5 min for antigen retrieval, cooled for 3 min and allowed to stay at room temperature for 60 min. After incubation in 3% H202 (3 $\times$ 15 min) to inhibit endogenous peroxidase activity, they were washed using 0.05 M Tris buffer (pH7.6) (3 $\times$ 5 min) and subsequently incubated with 2% normal bovine serum in Tris buffer for 10 min to block nonspecific activity. After incubation with normal serum, pre-treatment of the sections with Avidin D blocking solution for 15 min was followed by incubation with the biotin blocking solution in order to block nonspecific binding of Biotin/Avidin System reagents, according to the manufacturer's instructions (Vector Laboratories. Inc., Burlingame, CA). Then, the slides were incubated with rabbit polyclonal antibodies to *c-jun* (18) and *c-fos* gene products (22) (provided by Dr. H. Iba: Dept. of Gene Regulation. Inst. of Medical science, Univ. of Tokyo), and a c-fos gene product (K-25) (Santa Cruz Biotechnology, Inc.) for 12 hr at room temperature in a humidified atmosphere. K-25 broadly reacts with c-Fos, FosB, Fra-1 and Fra-2 of mouse, rat and human origin. After the slides were washed in Tris buffer (3 $\times$ 5 min), a Dako Labeled Strept-Avidin Biotin (LSAB) Kit was used to achieve the secondary and tertiary steps and complexes were visualized with diaminobenzine tetrahydrochroride (Dako, Carpinteria, IL) as a chromogen. The slides were washed in distilled water for 2 hr and counterstained using 1% methyl green solution for visual analysis of AP-1 protein immunostaining.

RESULTS

Expression of genes encoding AP-1 transcription factors. The nucleotide sequence of rat *c-fos* cDNA is about 82% homologous with that of the human *c-fos* cDNA according to nucleotide sequence homology data using Genetyx-Mac, 80.9% for *fra-1*, 89.1% for *fra-2*, 78.3% for *c-jun*, 84.2% for *junB*, and 76.9% for *junD*. Therefore, it can be inferred that the human AP-1 gene mRNAs were detected almost at the same efficiency as rat mRNAs under the conditions used in these experiments. Furthermore, we also used end-labeled oligonucleotides of *c-fos, fra2, junB* and *junD* containing a part of each rat homologue sequence and of fosB containing a part of mouse homologue sequence as probes in order to ensure mRNA expression coincided with the results for human cDNA probes (data not shown). To examine how expression of the AP-1 genes was related to renal carcinogenesis, we performed Northern blot analysis on four RC cell lines and RC tissues from Eker rats and normal kidney tissues from a non-Eker rat. The results are shown in Fig 1. Northern blot analysis revealed overexpression of *fra-1, fra-2, c-jun, junB* and *junD* mRNAs in all RC cell lines and tissues. mRNA for *c-fos* could not be detected, whereas a small amount of *fosB* mRNA was present limited to the RC cell line and tissue cases (data not shown).

TRE binding activities in RC cell lines. To analyze for TRE binding, we prepared crude nuclear extracts from an RC cell line (LK9d), one which typically expresses mRNAs of AP-1 genes, and performed electrophoretic mobility shift assays with a radiolabeled oligonucleotide containing a consensus TRE element. The results are shown in Fig. 2. The binding activity was competitively inhibited by 25 and 100-fold excess of cold oligonucleotide containing TRE consensus element, but a mutant TRE showed no competition, demonstrating that binding was specific for the TRE sequence. In addition, TRE binding was detected in RC cell lines from the Eker rats which we tested.

Immunostaining of AP-1 proteins. Immunohistochemistry of primary specific antibodies to c-jun and c-fos gene products (provided by Dr. Iba), diluted at 1:200 and 1:1000 respectively, demonstrated c-Jun in the nuclei of cells in phenotypically altered tubules and RCs (Fig. 3A and 3B). No c-Fos protein immunoreactivity was found. However, Fos family proteins were detected in the nuclei in phenotypically altered tubules and RCs with the commercial anti-c-Fos antibody which reacts with all Fos family members (K-25).

Suppression of LK9d cell growth by AP-1 gene antisense oligonucleotides. To determine whether reduction in the levels of AP-1 proteins and/or AP-1 DNA-binding activity might affect cell growth, the growth curves of LK9d cells treated with or without antisense oligonucleotides were compared. While no decrease in cell number was noted with individual antisense oligonucleotides (data not shown), the combination of all 7 brought about significant reduction (Fig. 4). Gel mobility-shift analysis of LK9d cells treated with this combination of AP-1 antisense oligonucleotides revealed reduced TRE binding activity as compared with the sense oligonucleotide case (Fig. 5).

DISCUSSION

Tumorigenesis is a complicated process associated with several changes in many genes involved in signal transduction. Therefore AP-1 proteins are believed to play important roles in this respect. Constitutively elevated AP-1 activity has been reported to be required for establishment and maintenance of the specific malignant phenotype in several cells (23), but it has hitherto been unclear whether activated AP-1 is required for renal carcinogenesis. The present study with the Eker rat model suggests that this may indeed be the case.

Thus overexpression of *fra-1, fra-2, c-jun, junB* and *junD* mRNAs in RCs as compared to normal kidney tissues was demonstrated by Northern blot analysis. In addition, both c-Jun and Fos family proteins were clearly detectable in RC cells and the earliest preneo-

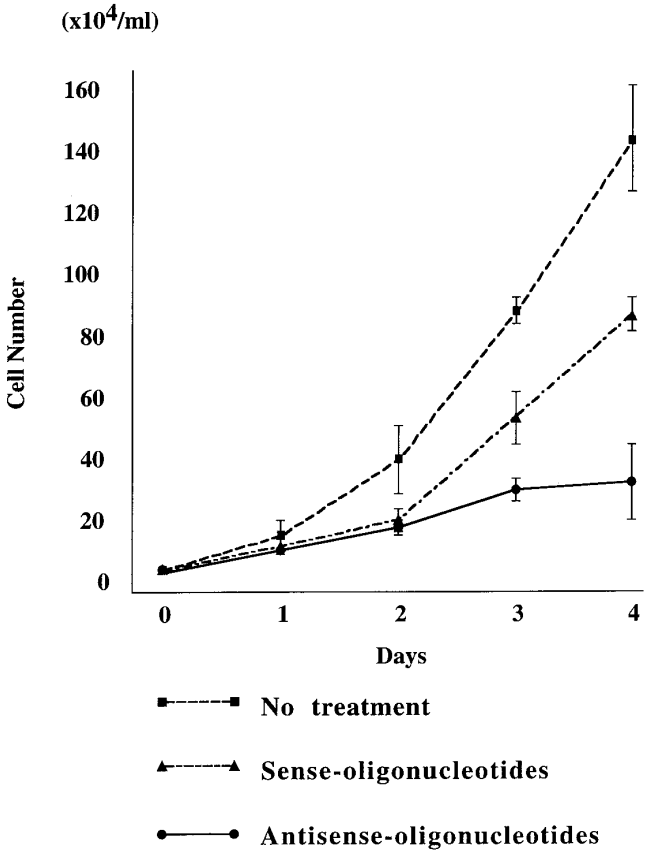


FIG. 4. Antiproliferative effect of antisense oligonucleotides for *jun* and *fos* gene family members on cell numbers of the LK9d renal carcinoma (RC) cell line (LK9d). Growth curves for LK9d cells are shown. LK9d cells were nontreated (■) or exposed to combinations control sense-oligonucleotides (▲) or antisense oligonucleotides for *jun* and *fos* gene family members(●). Values are means ± SD for triplicate dishes. The growth of cells treated by combination of the all antisense oligonucleotides was inhibited as compared with control sense oligonucleotides and no-treatment cases ($P<0.001$).

plastic cells (e.g., phenotypically altered renal tubules) by immunohistochemistry. These results raise the possibility that these AP-1 proteins play an important role in early stages of renal carcinogenesis in the Eker rat model. c-Jun and c-Fos have been reported to play a role in TPA induced tumor promotion in a two stage model of chemical carcinogenesis with mouse embryonic fibroblasts (24). AP-1 DNA binding activity is also known to be increased in malignant *vs* benign mouse keratinocytes (25). Induction of AP-1 genes may itself lead to further increase in the total amount of transcription through binding to the promoter region. In fact, *c-jun* transcription is well known to be directly stimulated by its own gene product because the *c-jun* promoter possesses a binding site for AP-1 (26). In addition to this upstream binding site, the *c-jun* gene contains at least one more AP-1 site down stream of the start of transcription, within the 5′ untranslated region (26). Thus a positive auto-regulatory loop is likely to be responsible for amplifying any transient signals generated (26). The overinduction of *fos* and *jun* family mRNAs leads to increased amounts of AP-1 proteins and we have shown AP-1 DNA-binding activity in RC cell lines. Elevated AP-1, which consists of homo- or heterodimers of Jun and/or Fos, may increase transcription of TPA response genes by binding to TRE recognition sequences within upstream promoter regulatory regions, including AP-1 genes themselves and other examples involved in growth control.

Suppression of Jun and Fos production by expression of antisense RNA or microinjection of antibodies has been reported to inhibit induction of cell proliferation and cell cycle progression (27-29). In this study, a combination of all the antisense oligonucleotides against

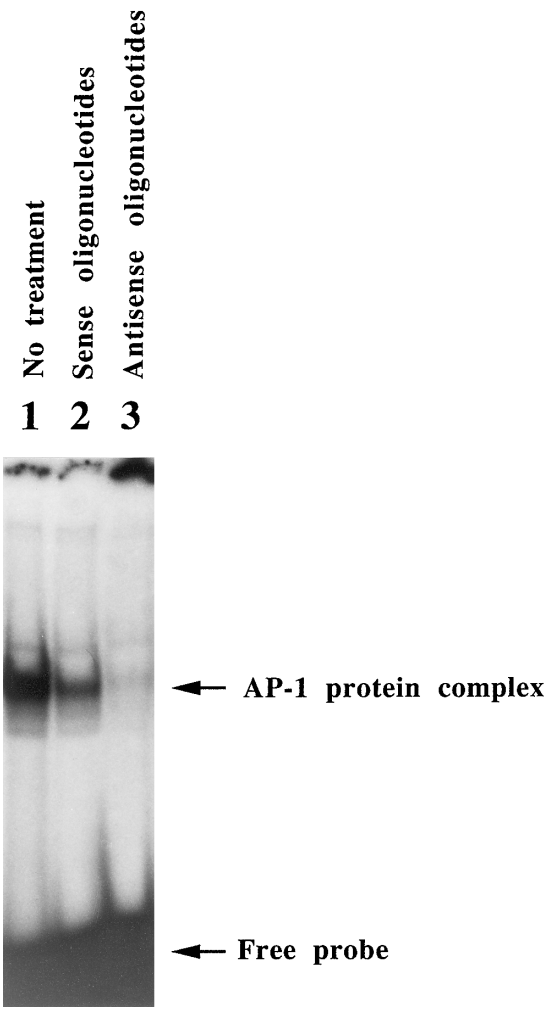


FIG. 5. AP-1-TRE binding activity in the renal LK9d carcinoma (RC) cell line treated with and without antisense oligonucleotides as assessed by electrophoretic gel mobility-shift assay. LK9d cells treated with antisense oligonucleotides (lane 3) demonstrated reduced TRE binding activity as compared with no-treatment (lane 1) or sense oligonucleotide treated cells (lane 2).

AP-1 genes similarly suppressed growth of an RC cell line from an Eker rat. The fact that single oligonucleotides were without effect suggests that AP-1 heterocomplexes rather than specific homo-complexes are related to proliferation of these cells.

Recently, we have demonstrated a *Tsc2* gene somatic mutation in a chemically-induced non-Eker rat RC (30), which also overexpressed AP-1 family members (data not shown). Our present finding calls attention to a possible role of *Tsc2* gene in the aberrant expression of AP-1 genes either directly or indirectly. Future search for down-regulation of AP-1 genes in RC cells with wild-type *Tsc2* gene expression using a tetracycline-responsive promoter system should be rewarding in this context. Finally, the results of the antisense oligonucleotide study may provide a basis for the design of new treatment strategies aimed at preventing AP-1 protein expression in RC cells.

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