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TISSUE-SPECIFIC *TRANS*-ACTIVATION OF RENIN GENE BY TARGETED EXPRESSION OF ADENOVIRUS E1A IN TRANSGENIC MICE

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SUMMARY: We generated two transgenic mice carrying the adenovirus type 12 E1 region genes under control of the human renin promoter, in which the E1A and E1B genes were expressed predominantly in the kidney. Interestingly, renal transcription of the gene for mouse renin was shown to be elevated at 28 and 40 folds as compared in those of control animals, but histon mRNA levels were unchanged. Although the transfected mouse renin promoter was promiscuously *trans*-activated by E1A in HeLa cells where the endogenous renin gene was silent, the transgenic studies suggested that a cellular factor(s), in addition to E1A, was required for the tissue-specific renin gene activation. These findings provide the *in vivo* evidence that E1A could *trans*-activate cellular gene transcription in a tissue-specific manner.

The study of complex cellular events, such as gene expression, is often facilitated by the use of simple viral systems along with cultured cell lines. Of these defined systems, animal viruses, especially the DNA tumor viruses, have been invaluable in developing an understanding of the basis of eukaryotic gene regulation. One such example is the study of transcriptional control by adenovirus (Ad) E1A gene. The Ad 12-type E1A transcription unit produces two major spliced mRNAs, the 12S and 13S forms, which encode proteins consisting of 235 and 266 amino acids, respectively. Generally, the larger E1A is thought to be a prototype transcriptional activator, and it stimulates transcription from the eukaryotic promoters and the five early Ad promoters such as those of E1B gene, even though they have no common *cis*-acting DNA sequence required for activation (1-3).

The most unusual property of E1A as a prototype *trans*-activator is its lack of specificity (3). Transcription from the cloned eukaryotic promoters is substantially stimulated by E1A, whether these promoters are introduced into E1A-expressing cells in the viral chromosome or in plasmids. In contrast with the promiscuous activation by E1A

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of many cellular promoters as episomal DNA, E1A-dependent activation of endogenous genes appears to occur infrequently (1-3). However, there is still less information available on the *in vivo* mechanism(s) by which E1A, when chromosomally integrated in the living animals, exerts its effect on cellular gene activation.

The introduction of cloned genes into mice has been proven to be a powerful tool to study the role of the respective gene products (4). Using this transgenic technique, we have recently demonstrated that the human gene encoded for renin, an aspartyl protease mainly produced in the kidney, along with the 3-kb 5'-flanking region is expressed predominantly in the kidney of transgenic mice (5, 6). In the present study, as a first step for monitoring the *in vivo* activity of E1A, we have directed expression of the Ad 12-type E1 region, including E1A and E1B genes, to the kidney of transgenic mice, under the control of the 3-kb human renin promoter. Using the mouse renin gene as an activation model, we demonstrated that the transfected mouse renin promoter *in vitro* is promiscuously *trans*-activated by Ad 12-type 13S E1A in the presence of cAMP but that the endogenous renin gene expression in transgenic mice carrying the Ad 12-type E1 region genes is stimulated in a tissue-specific manner.

MATERIALS AND METHODS

Cell Culture, DNA Transfection, and Chloramphenicol Acetyltransferase (CAT) Assay—HeLa cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). They were plated approximately 24 h before transfection at a density of 5×10^5 cells in 60-mm plastic dishes. Transient co-transfection of Ad 12-type 13S E1A- or 12S E1A-expression plasmids (7) with pmRn224CAT containing the mouse renin promoter (8) was performed using a $CaPO_4$ coprecipitation method essentially as described (9). Cells were harvested 48 h after transfection and assayed for CAT activity as described previously (10).

Construction of Fusion Transgene—A unique *Xho*I site of pMTVAd12 (11), in which the long terminal repeat (LTR) of mouse mammary tumor virus (MMTV) is fused to the complete Ad 12-type E1 region genes including those of E1A and E1B, was digested with *Xho*I and converted to the *Bgl*II site. The 5-kb *Bgl*II-*Eco*RI DNA fragment containing Ad E1 region genes was excised from the resulting plasmid and cloned into the *Bgl*II-*Eco*RI sites of pUC19 to make pE1AB/G. The 3-kb *Bam*HI DNA fragment containing the 5'-flanking region of the human renin gene was excised from pUChRNcat30 (12) and ligated into the *Bgl*II site of pE1AB/G to construct pUChRNE1AB.

Production of Transgenic Mice—For microinjection into fertilized eggs, pUChRNE1AB was cleaved with *Bam*HI and *Eco*RI. The 8-kb insert DNA was isolated by sucrose gradient centrifugation to remove the vector sequences and dialyzed against 10 mM Tris-HCl buffer (pH 7.5) and 1 mM EDTA. The DNA was used for directly for microinjection in a concentration of 4 μg/ml and about 1000 copies/embryo. One-cell zygotes fertilized in vitro were obtained from C57BL/6 mice, and outbred CD-1 females were used as the pseudo-pregnant recipients. The transgenic procedure used was essentially as described (13).

Reverse Transcription and Polymerase Chain Reactions—Total RNA was isolated from tissues of transgenic and age-matched sibling mice by the guanidine

thiocyanate/cesium chloride method (14). 0.5 μg of RNA were incubated with Molony Murine Leukemia Virus reverse transcriptase (M-MLV RT; BRL) for 60 min at 37°C in a total volume of 20 μl containing 1 x M-MLV RT buffer (50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 75 mM KCl), 0.5 mM of each dNTP, 20U RNasin (ToYoBo), and 100 pmol random primers. PCR (15) was performed in a thermal cycler (Perkin-Elmer Cetus) according to the following protocol: an initial denaturation at 94°C for 1 min was followed by 30 cycles of 1 min denaturation, annealing the primers at 60°C for 1 min, and extending the primers for 1 min at 72°C. Oligodeoxynucleotides representing nucleotide positions 849 to 868 (5'-TAGCGATTCGGAAGACGAGC-3', 5' end for E1A: A1), 1370 to 1351 (5'-ACATCTAGGGCGTTTCACTG-3', 3' end for E1A: A2), 3141 to 3160 (5'-TACTCGACATCGTTGCCGAC-3', 5' end for E1B: B1), and 3690 to 3671 (5'-CAGAATGTCCTCTCGAACTG-3', 3' end for E1B: B2) (16) were synthesized as primers by CYCLONE PLUS (DNA/RNA Synthesizer, MILLIPORE).

Northern Blot Analysis–Total RNA extracted from the kidney, liver, brain, and spleen of transgenic and sibling mice was denatured with 1 M glyoxal and 50% dimethylsulfoxide, electrophoresed on a 1.2% agarose gel, and transferred to GeneScreen Plus membrane (DuPont-New England Nuclear). Filters were hybridized to the ^{32}P-labeled 816-bp *Kpn*I/*Nco*I DNA fragment from mouse renin cDNA (17) or 693-bp *Hin*fI DNA fragment from mouse histon H4 gene (18) at 60°C for 16 h and washed twice with 2 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at room temperature for 5 min, twice with 2 x SSC/1% SDS at 60°C for 30 min, and twice with 0.1 x SSC at room temperature for 30 min.

RESULTS AND DISCUSSION

In order to test whether the mouse renin promoter was inducible by E1A, plasmid vectors expressing either Ad 12-type 13S E1A (pE1A12.13S) or 12S E1A (pE1A12.12S) and a recombinant containing the promoter region of the mouse renin gene linked to the CAT gene (pmRn224) were used for this assay. As the expression system, HeLa cells were used, in which the renin gene is not expressed. The mouse renin promoter region used in this experiments contains sequences between positions -224 to +16; our previous experiments show that this DNA fragment is responsible to cAMP in a kidney-derived cell line, but not in HeLa cells (19). To examine the effect of E1A on the activity of the mouse renin promoter, pmRn224 was transiently transfected with either pE1A12.13S or pE1A12.12S into HeLa cells in the absence or presence of cAMP. As shown in Fig. 1, although the CAT activity was induced neither by 13S E1A nor by cAMP alone, the renin promoter was *trans*-activated up to 26 folds only when given together. On the other hand, 12S E1A was unable to activate the mouse renin promoter, even in the presence of cAMP (data not shown). These results indicated that Ad 12-type 13S E1A but not 12S E1A was able to cooperate with cAMP to activate the mouse renin promoter *in vitro*, irrespective of its lack of the endogenous renin gene expression.

If renin responds to E1A in the presence of cAMP, then what is the physiological relevance of this observation with regard to the *in vivo* action of E1A ? The steady-state

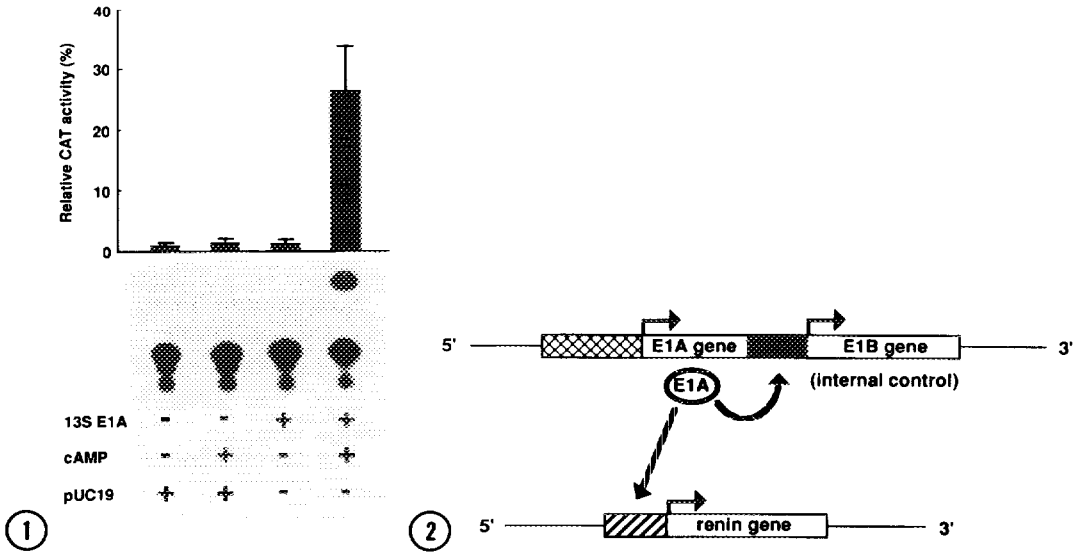

FIG. 1. ***Trans*-activation of mouse renin promoter by E1A.** Three micrograms of pmRn224CAT were co-transfected with 0.3 μg of pE1A12.13S (E1A13S) or pUC19 into HeLa cells. Twelve hours after transfection, the cells were incubated in the presence (+) or absence (-) of 1 mM 8-Br-cAMP for 48 h. Cell lysates were prepared and analysed for CAT activity.

FIG. 2. **Construct used in this study and *trans*-activation model by E1A.** The hRNE1AB gene contains 3-kb 5'-regulatory region of the human renin gene linked to the Ad 12-type E1 region without the native promoter of E1A gene. The cross-hatched, the filled, and hatched boxes represent the human renin promoter, the E1B promoter, and the mouse renin promoter, respectively. The thin lines represent mouse chromosome DNA. Expression of the E1B gene would serve as an *in vivo* control of *trans*-activation.

level of cAMP may vary with tissue type, thus resulting in different levels of cAMP-generating activity between tissues. As intracellular levels of cAMP are known to change in response to hormone or growth factor stimulation, one might expect that E1A probably can regulate renin transcription, depending upon tissue type. In fact, renin production in the kidney is shown to be influenced through β-adrenergic stimuli mediated by cAMP (20-22). In this respect, tissue culture experiments would not be suitable for examining the *in vivo* effect of E1A on cellular gene expression, since such systems cannot easily assess the influence of blood supply, hormone and growth factors—all elements presumed crucial to efficient gene transcription *in vivo*. Therefore, we have turned from the cultured cell system to the transgenic mouse, a system in which the action of E1A can be assessed in a living host capable of mounting physiologic response to renin transcription, and chosen the kidney as a target tissue to investigate the *in vivo* transcriptional activity of E1A.

For this purpose, the DNA construct used to generate the transgenic mice was specifically designed to express biologically active E1A as follows: First, to target the expression of Ad 12-type E1A gene to the kidney, we have subjected its genomic DNA to

the 3-kb 5'-regulatory region of the human renin gene since these sequences played a major role in expressing the renin gene predominantly in the kidney of transgenic mice (5, 6, 12); Second, Ad 12-type E1B gene along with its native promoter was linked immediately downstream to the renin promoter/E1A fusion gene. The E1B transcriptional unit would serve as an *in vivo* control of *trans*-activation (Fig. 2), because a detailed genetic study indicated that E1A *trans*-activates the E1B promoter (23).

The 8-kb hRNE1AB DNA fragment (Fig. 3A) was isolated and microinjected into the pronuclei of one-cell C57BL/6 embryo. Mice from the resulting litters were tested for the presence of the transgene by Southern blot hybridization. Two positive mice, E1AB72 (male) and E1AB73 (female), were generated, both of which carried the 1 copy of transgene (data not shown) and used for further analyses one month after birth, because of their delayed growth as compared with that of its siblings. Therefore, no offspring could be obtained from them.

Total RNA was prepared from kidney, liver, lung, spleen, and brain, and analyzed for expression of the transgene and the endogenous renin gene by RT-PCR and Northern hybridization, respectively. The PCR product for Ad 12-type E1A 13S mRNA can be distinguished from that for 12S mRNA on a polyacrylamide gel as they differ in size by 93 bp. As shown in Fig. 3B, the both mRNA products in E1AB72 and E1AB73 mice were

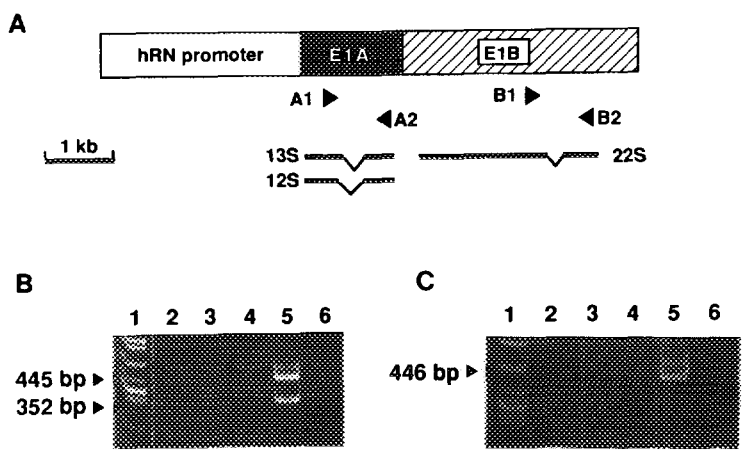

FIG. 3. **Detection of transgene mRNAs in mice by RT-PCR.** *A*. Structure of the renin-E1AB transgene. The arrowheads represent the locations and orientations (5' to 3') of the primers of E1A (A1 and A2) and E1B (B1 and B2) genes. *B* and *C*. Representative ethidium bromide-stained polyacrylamide gels of RT-PCR products with specific primers for E1A (panel *B*) and E1B (panel *C*). Lane 1 contains ϕX174-*Hin*cII digested DNA marker. Lanes 2 to 6 contain RT-PCR products using RNA isolated from E1AB72 liver, lung, spleen, kidney and brain, respectively. The arrowheads at 445 bp, 352 bp, and 446 bp indicate the positions of RT-PCR products for 13S E1A, 12S E1A, and 22S E1B, respectively.

expressed predominantly in the kidney, with lower levels found in the brain, while they were undetectable in the liver, lung and spleen. Furthermore, the PCR product for E1B mRNA was detected only in the kidney and brain (Fig. 3C). These results indicated that Ad 12-type E1A gene products can activate transcription from the E1B promoter, even in the integrated chromosome of mice, suggesting that the expression of E1B gene is determined by E1A. The extra bands other than the expected products observed in Fig. 3B and 3C may be amplified by PCR from a genomic DNA contaminated in RNA samples during extraction or from transcriptionally immature RNA.

Finally, we examined whether the targeted expression of Ad 12-type E1A gene to the kidney affects expression of the mouse renin gene in the two transgenic mice. As shown in Fig. 4, Northern blot analysis revealed that the levels of renin mRNA were increased up to 28 and 40 folds as compared with those of age-matched siblings, while no renin mRNA level was augmented in the brain. On the other hand, the levels of histon H4 mRNA were unchanged. This unusual activation of the renal renin gene might result in the combined effects of E1A and cAMP, and is likely to be consistent with the *in vitro* synergistic action of both effectors on *trans*-activation of the mouse renin promoter (Fig. 1). These results suggested that the endogenous renin gene expression in transgenic mice could be stimulated by E1A in a tissue-specific manner.

E1A does not appear to activate transcription by binding to specific DNA sequences since no specific motif is required for E1A activation (24) and since purified E1A protein

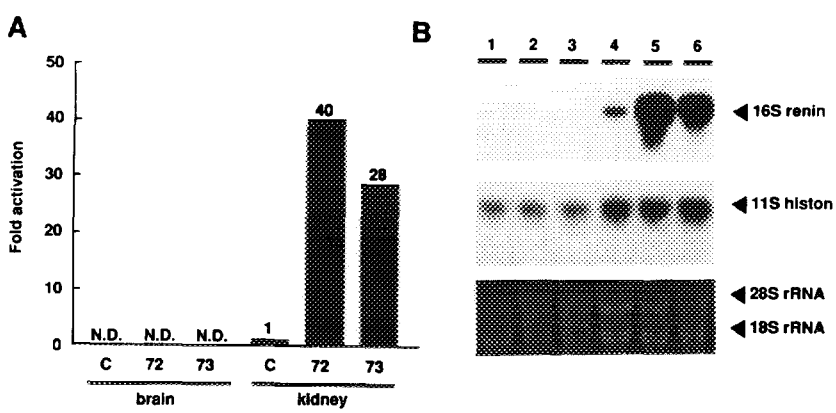

FIG. 4. **Activation of cellular renin gene expression.** *A*. Renin mRNA levels shown in panel *B* were determined by scanning of the resulting autoradiogram, using BioImage analyzer (MILLIPORE), normalized to the intensity of mouse histon mRNA assayed as an internal control. *B*. Twenty micrograms of total RNA were assayed for renin and histon expression. The upper and middle panels show the blots hybridized to the mouse renin and mouse histon probes, respectively. The lower panel indicates the ethidium bromide-stained 18S and 28S ribosomal RNA as a control of the amount of RNA applied (panel B). Lanes 1 to 3, brain; 4 to 6, kidney. Lanes 1 and 4, control; 2 and 5, E1AB72; 3 and 6, E1AB73. N.D.; not detected.

does not bind to DNA with sequence specificity (25). Rather, E1A-induced *trans*-activation most likely occurs through stimulation of other transcription factors (1-3). Taken together with these observations, in contrast with the present *in vitro* results that the transfected mouse renin promoter is promiscuously *trans*-activated by E1A in cultured HeLa cells (Fig. 1), our transgenic studies suggest that a tissue-specific cellular factor(s), in addition to E1A, is required for the renin gene activation *in vivo* (Fig. 4).

Prior to the present study, the known *trans*-activation effects of E1A were limited to cell culture studies *in vitro*, and thus action of the chromosomally integrated E1A on gene expression in the living animals was poorly understood. We have now extended such *in vitro* studies to show that E1A has an *in vivo* action on cellular gene activation. The transgenic mice containing the Ad 12-type E1 region genes demonstrate *in vivo* the tissue-specific stimulatory effect of E1A on the renin gene expression. This type of approach described here will be applicable to study further the molecular regulation of E1A-responsive genes and their biological action *in vivo*.

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