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Induction of Renal Adenocarcinoma by a Nonmutated *erbB* Oncogene

C. A. TAGLIENTI-SIAN,[1] B. BANNER,[2] R. J. DAVIS,[3,4] AND H. L. ROBINSON[1,2]*

Department of Molecular Genetics and Microbiology,[1] Department of Pathology,[2] Howard Hughes Medical Institute,[3] and Program in Molecular Medicine,[4] University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655

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Oncogenicity tests have revealed that a nonmutated *erbB* oncogene induces renal adenocarcinoma in addition to erythroblastosis. The *erbB* oncogene is a truncated form of the chicken epidermal growth factor receptor that lacks the extracellular ligand-binding domain. Previously, the nonmutated *erbB* oncogene has been reported to cause only erythroblastosis. The expansion of the disease potential of *erbB* to additional neoplasms has been associated with mutations (truncations, deletions, and point mutations) within the *erbB* gene. Our results indicate that a nonmutated virally expressed *erbB* oncogene (REB-c) causes a 100% incidence of renal neoplasia.

The *erbB* oncogene encodes the transmembrane, protein tyrosine kinase, and C-terminal autophosphorylation domains of the epidermal growth factor (EGF) receptor (for a review, see reference 15). Nonmutated forms of the *erbB* oncogene cause erythroblastosis in chickens (1, 11, 20, 21, 25, 33). Point mutations in the kinase domain can broaden this disease potential to the induction of fibrosarcoma (29, 30). Truncations, in-frame deletions, and point mutations in the C-terminal domain extend the *erbB* disease potential to the induction of angiosarcoma and fibrosarcoma (11, 15, 23, 25, 29, 30, 32). The oncogenic potential of *erbB* can also be increased by the presence of another oncogene. This is best exemplified by avian erythroblastosis virus ES4 (AEV-ES4) and AEV-R, viruses that contain both the *erbB* and the *erbA* oncogenes (6, 10, 14, 27). AEV-ES4 has been reported to cause renal adenocarcinoma as well as erythroblastosis and fibrosarcoma (3; for a review, see reference 15). td359, a variant of AEV-ES4 that is defective for *erbA* and contains a C-terminal truncation of *erbB*, causes renal adenocarcinoma and fibrosarcoma in the absence of erythroblastosis (6, 18). Here we show that a nonmutated *erbB* can cause renal adenocarcinoma as well as erythroblastosis.

The disease potential of *erbB* oncogenes has been evaluated by using avian leukosis virus vectors. Many of these have represented naturally occurring transductions of *erbB* (1, 6, 10, 16, 23, 28, 34). More recently, *erbB* sequences have been expressed by using replication-competent avian leukosis virus vectors (e.g., RCAN) (13, 20, 21, 29, 30). In particular, a construct designated REB-c has been used to test for the oncogenic potential of mutations in *erbB* (20, 21, 29, 30). The parent REB-c vector expresses a nonmutated, C-terminal complete *erbB* (21). Our studies were conducted with a modified REB-c which contains *gag* and *pol* sequences from the Bryan high-titer strain of Rous sarcoma virus (RCANBGP) (13, 29). REB-c causes a high incidence of erythroblastosis and a moderately low incidence of wing web tumors (20, 21, 29, 30, 32).

Tests of the oncogenic potential of REB-c revealed a 100% incidence of renal adenocarcinoma in addition to a 100% incidence of erythroblastosis and a 33% incidence of wing web tumors (Fig. 1). Both 1-day-old specific-pathogen-free SPAFAS (SPAFAS, Norwich, Conn.) and K28 (24) chicks were used for inoculations. Oncogenicity tests were initiated by inoculation of (i) pREB-c-transfected chicken embryo fibroblasts in virus-containing culture medium, (ii) culture medium from transfected chicken embryo fibroblasts, or (iii) REB-c plasmid DNA. Chickens were inoculated intravenously, intraperitoneally, and in the wing web. Inoculations of cells plus virus consisted of $\sim10^6$ cells and 0.1 to 0.2 ml of culture medium per site. Virus inoculations consisted of 0.1 to 0.2 ml of culture medium per site. For DNA inoculations, 100 μg of cesium chloride-purified plasmid DNA in 0.05 to 0.2 ml of saline was used at each inoculation site. Use of direct DNA inoculations eliminates the occurrence of mutations in *erbB* during the recovery of virus in cell cultures. Beginning at 1 to 2 weeks postinoculation, chicks were monitored twice weekly for the presence of erythroblasts in blood smears and signs of wing web tumors. Chickens were terminated at approximately 3 to 6 weeks postinoculation, a time when all of the chicks (inoculated with either cells plus virus, virus, or DNA) had developed erythroblastosis. Necropsy was performed, and tissues were fixed in phosphate-buffered formalin and processed for histologic analyses. Renal tumors were first noticed at the time of autopsy. Kidney tumors were observed whether infected cells plus virus, virus, or DNA was used as the inoculum. Control chicks inoculated with vector DNA (pRCANBGP) alone became viremic but did not develop disease.

Histologic evaluation of the REB-c-induced renal tumors revealed a spectrum of changes from hyperplasia of the tubular epithelium to invasive adenocarcinoma (Fig. 2). Proliferative epithelial lesions were most prominent in the subcapsular region of the kidney and created a lacelike appearance in low-power-microscopic sections (Fig. 2A). The epithelial lesions were classified as hyperplasia if there was hypercellularity within single tubules; adenoma if in addition to hypercellularity there were architectural abnormalities such as papillary infoldings, cystic change, or an increase in the number of tubules to form a nodule (Fig. 2B); and adenocarcinoma if marked cytoplasmic abnormalities or stromal invasion was noted (Fig. 2C). All kidneys exhibited hyperplasia and adenoma. Features of malignancy (adeno-

* Corresponding author.

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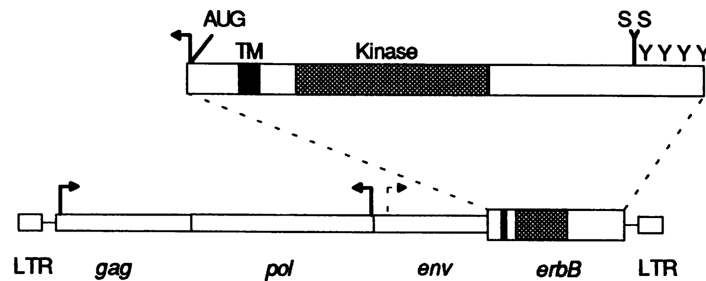
A.



B.

Construct	Inoculum	Renal Adenoma/ Adenocarcinoma	Erythroblastosis	Wing Web Tumors
REB-c	DNA	12/12	12/12	4/12
	Virus	1/1	1/1	0/1
	Cells + Virus	1/1	1/1	1/1
REB-c Ala[477/8]	DNA	7/7	7/7	5/7
	Virus	2/2	2/2	2/2
	Cells + Virus	2/2	2/2	2/2
REB-c Glu[477/8]	DNA	2/2	2/2	1/2
	Cells + Virus	3/3	3/3	3/3
RCANBGP	DNA	0/6	0/6	0/6

FIG. 1. Induction of renal neoplasia by REB-c. (A) Schematic representation of REB-c. ◄┐, splice acceptor; ┌►, splice donor; ┌►, cryptic splice donor; AUG, start codon; TM, transmembrane domain; Kinase, protein tyrosine kinase domain; SS, serine 477/478 phosphorylation site; Y, tyrosine autophosphorylation site; LTR, long terminal repeat. (B) The incidence of renal adenoma or adenocarcinoma, erythroblastosis, and wing web tumors is presented as the ratio of number diseased to number inoculated.

carcinoma), including stromal invasion, and cytologic changes of increased nuclear-cytoplasmic ratio and nuclear pleomorphism were present in ~50% of the kidneys. The proliferating epithelial cells appeared to be in the distal convoluted tubule or collecting ducts, as the proximal tubules and glomeruli were normal. The histologic characteristics of the tumors were comparable to those reported for the AEV-ES4- and td359-induced renal cell tumors (3, 18).

Mutations at the serine 477/478 phosphorylation site of *erbB* that enhance the ability to induce wing web tumors (32) did not significantly affect the histologic characteristics of the renal adenocarcinoma (Fig. 1). Mutational removal of Ser-1046/1047, the human EGF receptor homolog of serine 477/478, has demonstrated that phosphorylation at this site acts to suppress EGF-induced signal transduction (5, 31). Ala-477/478 and Glu-477/478 mutations in *erbB* have been described previously (32). Transfected cells plus virus, virus, and plasmid DNA for both of these mutant *erbB* genes caused adenocarcinoma with histologic characteristics similar to those induced by the nonmutated C-terminal complete *erbB* (REB-c).

Internal deletions and truncations in C-terminal *erbB* sequences have previously been associated with the broadening of the oncogenic potential of *erbB* (11, 15, 23, 25, 30). Therefore, the *erbB* genes from two adenocarcinomas were analyzed to examine whether the 3′ *erbB* sequences were the same size as the parent REB-c vector. The 3′ region bounded by *Bam*HI and *Hin*dIII (Fig. 3A) has been a common target for deletions and truncations (6, 11, 15, 23, 25, 30, 36, 37). To test for the presence of deletions and truncations, DNA was extracted from diseased kidneys and from uninfected control kidneys. With the kidney DNA as a template, oligonucleotides 5′ to the *Bam*HI site and within the long terminal repeat of REB-c were used for amplification of a 1.27-kb REB-c fragment (Fig. 3A). The polymerase chain reaction (PCR)-amplified DNA was digested with *Hin*dIII to demonstrate that the 1.27-kb product contained the expected 882-bp *erbB* fragment. Both cut and uncut PCR products were run on 2% agarose gels (3 parts NuSieve agarose and 1 part SeaKem agarose; FMC, Rockland, Maine), transferred to nitrocellulose, and probed with an *erbB*-specific probe generated by random priming. Ethidium

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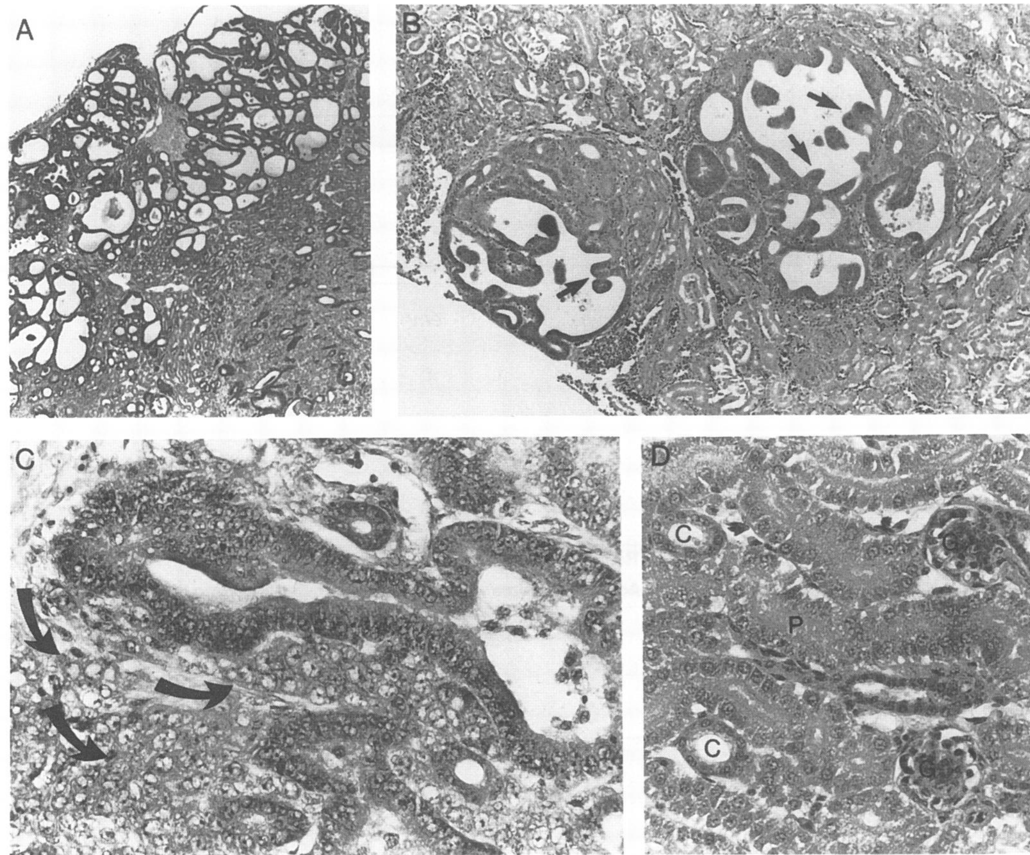

FIG. 2. Microscopic sections of kidneys showing the pathologic patterns in REB-c-induced renal adenoma or adenocarcinoma. (A) Lacelike appearance of REB-c-induced neoplasia (hematoxylin and eosin stain [HE]; magnification, ×20). (B) Adenomatous lesions characterized by architectural distortion with formation of two nodules and papillary infoldings of the epithelium (shown by arrows) (HE; magnification, ×100). (C) Adenocarcinoma characterized by a nodule exhibiting a focus of stromal invasion (shown by arrows). Invading cells have an increased nuclear-cytoplasmic ratio (HE; magnification, ×400). (D) Normal, uninfected control kidney. C, collecting ducts; G, glomeruli; P, proximal tubules (HE; magnification, ×400).

bromide-stained gels (data not shown) and autoradiographs (Fig. 3B, lanes 3 and 4) demonstrated that a single fragment of approximately 1.27 kb was amplified from the diseased kidneys. This fragment was the same size as that amplified from control REB-c plasmid DNA and contained the expected internal *Hin*dIII site (Fig. 3B, lanes 5 to 6 and 12 to 14). No bands were observed in the amplification reactions of DNA isolated from normal uninfected kidneys (Fig. 3B, lanes 1 and 2). This analysis demonstrates that the *erbB* DNA in the diseased kidneys did not contain detectable deletions in 3′ coding sequences. This analysis does not rule out the presence of activating point mutations. However, it is unlikely that *erbB* sequences in 100% of the test chickens would have acquired point mutations that activated the potential of *erbB* to cause renal adenocarcinoma.

Very little has been reported about the disease status of kidneys from *erbB*-infected birds in previous experiments. Two *erbB* viruses, AEV-ES4 and td359, have been shown to induce renal adenocarcinoma (3, 18). Both of these *erbB* viruses contain mutations (4, 6) that have been demonstrated to cause broadening of *erbB* disease potential (30). Retrospective analysis of histologic sections from our prior oncogenicity tests revealed the induction of a low incidence of renal neoplasia (adenoma) by a spectrum of *erbB* viruses. These included viruses both with activating mutations (AEV-H, avian angiosarcoma virus 5005, and avian angiosarcoma virus 4890 [11, 25, 37]) and without activating mutations (AEV-5005 [11, 25]). This suggests that most *erbB* genes have the potential to cause renal neoplasia. REB-c-infected chickens exhibited a higher incidence and more advanced stages of neoplasia (adenocarcinoma) than those in previous oncogenicity tests. This increased disease potential may be a result of the more efficient spread of the *erbB* oncogene in a replication-competent vector. In our previous tests, *erbB* was expressed by defective retroviruses that required the presence of a coinfecting helper virus for transmission.

The kidney is a major site of EGF synthesis in the body, and EGF receptors have been demonstrated in various cells of renal origin (2; for reviews, see references 8 and 12). Although the roles of EGF and the EGF receptor in the kidney are unclear, it is likely that these molecules have an important function in renal physiology (for reviews, see references 8 and 12). Perturbation of the EGF receptor or its ligands may have a role in neoplastic transformation in the kidney. There are numerous reports in which human renal cell carcinomas demonstrate abnormally high levels of expression of the EGF receptor (9, 17, 22, 26, 35, 38). In

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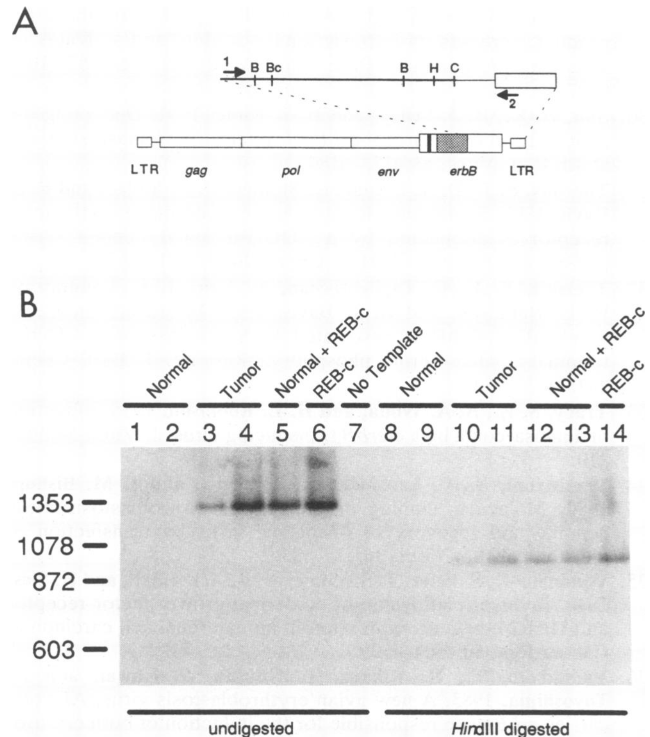

FIG. 3. PCR and Southern blot analysis of REB-c-induced renal adenocarcinoma. (A) Diagram of REB-c and its 3' structure. B, *Bam*HI; Bc, *Bcl*I; H, *Hin*dIII; C, *Cla*I; LTR, long terminal repeat. Oligonucleotides used for PCR analysis are designated 1-5' AGT GATGTCTGGAGTTATGGTGTGACA 3' and 2-5' CTCCTTGT AAGGCATGTTGCTAAC 3'. (B) Southern blot analysis of diseased and uninfected control kidneys. Genomic DNAs were prepared from kidney tissue, digested with *Stu*I, and amplified by PCR. The PCR products were digested with *Hin*dIII, and both digested and undigested products were analyzed by Southern blotting as described in the text. Filters were hybridized to a ^{32}P-labelled random-primed probe generated from a PCR-amplified fragment of REB-c (*Bam*HI to *Hin*dIII). Lanes: 1, 2, 8, and 9, 1 μg of DNA from kidneys of two different uninfected chicks; 3, 4, 10, and 11, 1 μg of DNA from the kidneys of two different REB-c-infected chicks; 5, 12, and 13, 100 pg of pREB-c amplified in the presence of 1 μg of DNA from uninfected kidneys; 6 and 14, 100 pg of pREB-c DNA; 7, PCR mixture containing no template. φX174-*Hae*III molecular weight markers are as indicated.

addition, other epithelial malignancies have been shown to express high levels of the EGF receptor (7, 19). The demonstration that a virally expressed, truncated form of the chicken EGF receptor (*erbB*) can cause renal adenocarcinoma (Fig. 1 and 2) is consistent with the hypothesis that perturbation of the EGF receptor may have a role in neoplastic transformation of the kidney.

In summary, we have demonstrated a new disease potential for the nonmutated *erbB* oncogene. Specifically, we have shown that a virally expressed C-terminal complete *erbB* induces a 100% incidence of renal neoplasia.

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