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A v-*erbB*-related protooncogene, c-*erbB-2*, is distinct from the c-*erbB-1*/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma

(*src* gene family/kinase family/gene amplification/cancer)

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ABSTRACT **From a human genomic library, we obtained six v-*erbB*-related DNA clones. A DNA probe prepared from one of the clones, λ107, hybridized to *Eco*RI fragments of 6.4 and 13 kilobase pairs of human DNA. Neither of these fragments was amplified in A431 vulva carcinoma cells, in which the gene encoding the epidermal growth factor receptor is amplified. In addition, the probe from λ107 hybridized with a single, 4.8-kilobase poly(A)$^+$ RNA species and did not react with EGF receptor mRNA. Thus, we conclude that clone λ107 represents a v-*erbB*-related gene (c-*erbB-2*) that is distinct from the EGF receptor gene. In contrast, the other five clones were shown to represent the EGF receptor gene (c-*erbB-1*). Partial nucleotide sequence analysis of the λ107 insert showed that this clone contained at least seven putative exons and that six of them could encode the kinase domain characteristic of protein products of the *src* oncogene family. Southern blot analysis showed close similarity of the restriction patterns of the rat c-*erbB-2* gene and the rat *neu* oncogene, suggesting possible involvement of c-*erbB-2* in human cancer. In fact, ≈30-fold amplification of c-*erbB-2* was observed in a human adenocarcinoma of the salivary gland.**

At least 19 genes have been identified as retroviral oncogenes that are responsible for inducing tumors *in vivo* and transforming cells *in vitro* (1). Ten of them apparently encode transforming proteins that share a kinase domain homologous to that of pp60^{src}, a tyrosine-specific protein kinase. The cellular cognate, encoded by the c-*src* gene, also exhibits tyrosine-specific kinase activity. Of particular interest is the fact that tyrosine-specific kinases are also encoded by the genes for several receptors for polypeptide growth factors, including the receptors for epidermal growth factor (EGF) (2), platelet-derived growth factor (PDGF) (3), insulin (4), and insulin-like growth factor I (5). This implies a possible link between the action of the growth factor–receptor complex and the oncogene product with tyrosine-specific kinase activity. In fact, recent analysis of the v-*erbB* gene and the EGF receptor gene indicated that the v-*erbB* gene is a part of the EGF receptor gene and codes for the internal domain and transmembrane portion of the receptor (6–8). These findings, together with the extensive identity of the amino acid sequences of the v-*sis* protein and platelet-derived growth factor (9, 10), suggest that some viral oncogene products mimic the action of the polypeptide growth factor–receptor complex in activating a cellular pathway involved in cell proliferation.

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We examined details of the relation between the v-*erbB* gene and the EGF receptor gene and the possible involvement of this gene in human cancer. During this study we identified two v-*erbB*-related genes, c-*erbB-1* and c-*erbB-2*, in the human genome.

MATERIALS AND METHODS

Cells and Tissues. A431 vulva carcinoma cells and human embryo fibroblasts were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. K562 chronic myelogenous leukemia cells and MT2 adult T-cell leukemia cells were maintained in RPMI 1640 medium with 10% fetal calf serum. Mouse FM3A cells were from M. C. Yoshida (Hokkaido University) and primary tumors were provided by K. Rikimaru (Tokyo Medical and Dental University).

Isolation of Clones. A human genomic library was constructed from placental DNA as described (11). The library was screened for the v-*erbB*-related sequence by plaque-hybridization as described (12) in 30% (vol/vol) formamide/4× NaCl/Cit (1× is 0.15 M NaCl/15 mM sodium citrate)/50 mM Hepes, pH 7.0/denatured salmon sperm DNA (20 μg/ml)/10× Denhardt's solution (1× is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin) at 42°C for 16 hr. After hybridization, the filters were washed with 2× NaCl/Cit/0.1% NaDodSO$_4$ at room temperature and then with 0.6× NaCl/Cit/0.1% NaDodSO$_4$ at 50°C. Plaques were purified by successive plaque-hybridization. The probe used was a 1.7-kilobase-pair (kbp) *Sst* I–*Stu* I DNA fragment that represents the v-*erbB* gene of avian erythroblastosis virus strain H (6); it was labeled with [α-^{32}P]dCTP by nick-translation (13) to a specific activity of 2 × 10^8 cpm/μg of DNA. Phage DNAs were prepared as described (14).

Nucleotide Sequence Analysis. The nucleotide sequence was determined by the procedure of Maxam and Gilbert (15) and the dideoxy chain-termination method (16, 17) in conjunction with bacteriophage M13 mp19 (18).

Blot-Hybridization Analysis of DNA and RNA. High molecular weight DNAs were prepared from chicken blood, human embryo fibroblasts, mouse FM3A cells, rat spleen, and primary tumors. The DNAs (10 μg per lane) were digested with restriction endonucleases under the conditions recommended by suppliers (Takara Shuzo) and fractionated by electrophoresis in 1% agarose gels. The fragments were subjected to Southern blot hybridization (19) at 42°C for 16 hr in 4× NaCl/Cit/50 mM Hepes, pH 7.0/10× Denhardt's solution/denatured salmon sperm DNA (20 μg/ml)/30% (relaxed conditions) or 50% (stringent conditions) formamide. After hybridization, the filters were washed with 2×

Abbreviations: EGF, epidermal growth factor; kb, kilobase(s); kbp, kilobase pair(s).

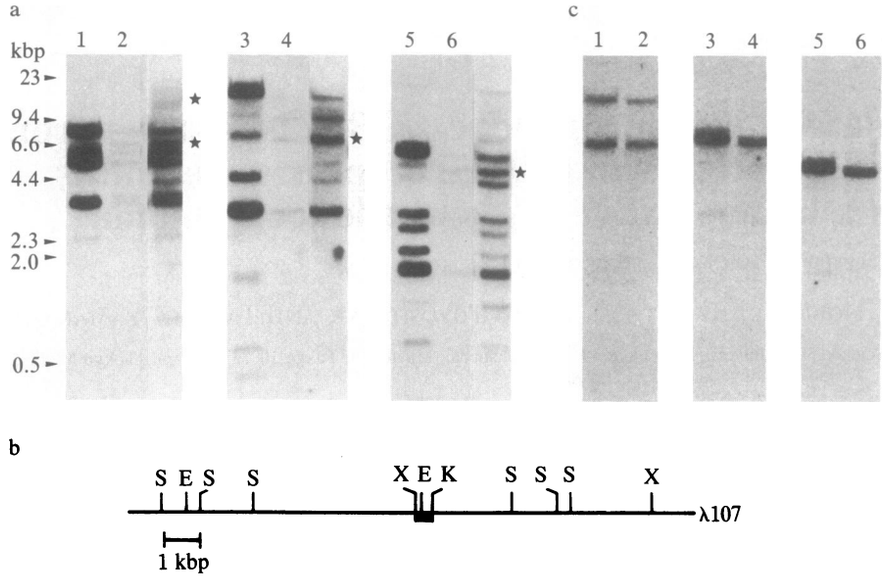

FIG. 1. Identification and cloning of the v-*erbB*-related sequence that is distinct from the EGF receptor gene. (*a*) Hybridization with the v-*erbB* probe. DNAs prepared from A431 cells (lanes 1, 3, and 5) and placenta (lanes 2, 4, and 6) were digested with *Eco*RI (lanes 1 and 2), *Sac* I (lanes 3 and 4), or *Pvu* II (lanes 5 and 6). Because the placental DNA was contaminated with RNA, samples in lanes 2, 4, and 6 contained less DNA than those in lanes 1, 3, and 5. The digests were subjected to Southern blot hybridization using a v-*erbB* probe under the relaxed conditions described in *Materials and Methods*. Autoradiographs obtained by longer exposures of lanes 2, 4, and 6 are shown to the right of the respective lanes. Arrowheads indicate positions of fragments of *Hin*dIII-digested λ DNA used as standards. Stars indicate bands that were not amplified in the A431 samples. (*b*) Restriction map of the λ107 insert. E, K, S, and X represent restriction sites of *Eco*RI, *Kpn* I, *Sac* I, and *Xba* I, respectively. The thick line indicates the *Kpn* I–*Xba* I fragment (KX fragment) used as a specific probe of the c-*erbB-2* gene. (*c*) Hybridization with the KX fragment. The same filter represented in *a* was washed and then hybridized with the KX probe under the stringent conditions described in *Materials and Methods*.

NaCl/Cit/0.1% $NaDodSO_4$ and then with either 0.2× NaCl/Cit/0.1% $NaDodSO_4$ (stringent) or 0.6× NaCl/Cit/0.1% $NaDodSO_4$ (relaxed). RNAs were prepared by the guanidinium isothiocyanate/cesium chloride method (20). Poly(A)$^+$ RNA selected by oligo(dT)-cellulose (P-L Biochemicals type 7) column chromatography was denatured with 50% formamide/2.2 M formaldehyde and 5 μg of each RNA sample was subjected to electrophoresis in 1% agarose gel containing 2.2 M formaldehyde (21). RNAs on the gel were transferred directly to a nitrocellulose filter and subjected to blot hybridization under stringent conditions, as described (22). The DNA probes used for blot hybridization were v-*erbB* DNA (described above), an EGF receptor cDNA clone (pE7) (22), and c-*erbB-2*-specific DNA (described below).

RESULTS

Isolation of a Human v-*erbB*-Related Sequence Distinct from the EGF Receptor Gene. High molecular weight DNAs prepared from A431 cells and human placenta were digested with either *Eco*RI, *Sac* I, or *Pvu* II, and the digests were analyzed by Southern blot hybridization under relaxed conditions with the v-*erbB* probe, which covers almost 90% of the v-*erbB* gene. Most of the v-*erbB*-related sequences were amplified in A431 cells at least 20-fold compared with those in placenta (Fig. 1*a*). These sequences were observed as 8.1-, 5.9-, 5.4-, and 3.5-kbp *Eco*RI fragments; 20-, 4.7-, 3.3-, 1.7-, 0.9-, and 0.5-kbp *Sac* I fragments; and 6.6-, 3.3-, 2.9-, 2.3-, 1.9-, and 0.9-kbp *Pvu* II fragments. The amplified sequences were of the EGF receptor gene as reported (8, 23). However, we observed several restriction fragments that hybridized to the v-*erbB* probe but that were not amplified in A431 cells (13- and 6.4-kbp *Eco*RI fragment, a 6.9-kbp *Sac* I fragment, and a 5.1-kbp *Pvu* II fragment, marked with stars in Fig. 1*a*). This suggested that besides the EGF receptor gene, there may be another v-*erbB*-related gene, from which the unamplified restriction fragments are generated.

We searched a human genomic library for v-*erbB*-related sequences, using the v-*erbB* probe, under the relaxed hybridization and washing conditions, and isolated six independent clones. Restriction map analysis showed that all the cloned inserts except the λ107 insert represented one gene, c-*erbB-1*. In these clones, we identified four *Eco*RI fragments as exon-containing sequences by the criterion that they hybridized to the v-*erbB* probe. All these fragments were

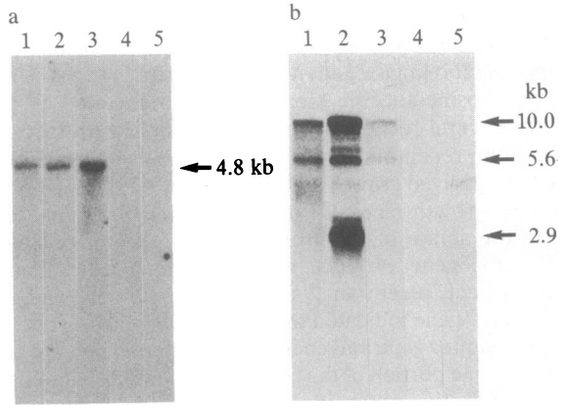

FIG. 2. Expression of the EGF receptor gene and the c-*erbB-2* gene in human cells. Duplicate samples of poly(A)$^+$ RNA were subjected to blot hybridization with the ^{32}P-labeled KX fragment (*a*) or with the ^{32}P-labeled EGF receptor cDNA clone pE7 (22)(*b*). Sizes of RNA species that hybridize with the probes are given. Chicken ribosomal RNAs (28S, about 4.8 kb, and 18S, about 2.0 kb) and Rous sarcoma virus RNA (39S, about 10.0 kb) served as size standards. RNAs were isolated from human placenta (lanes 1), A431 cells (lanes 2), human embryo fibroblasts (lanes 3), MT2 cells (lanes 4), and K562 cells (lanes 5).

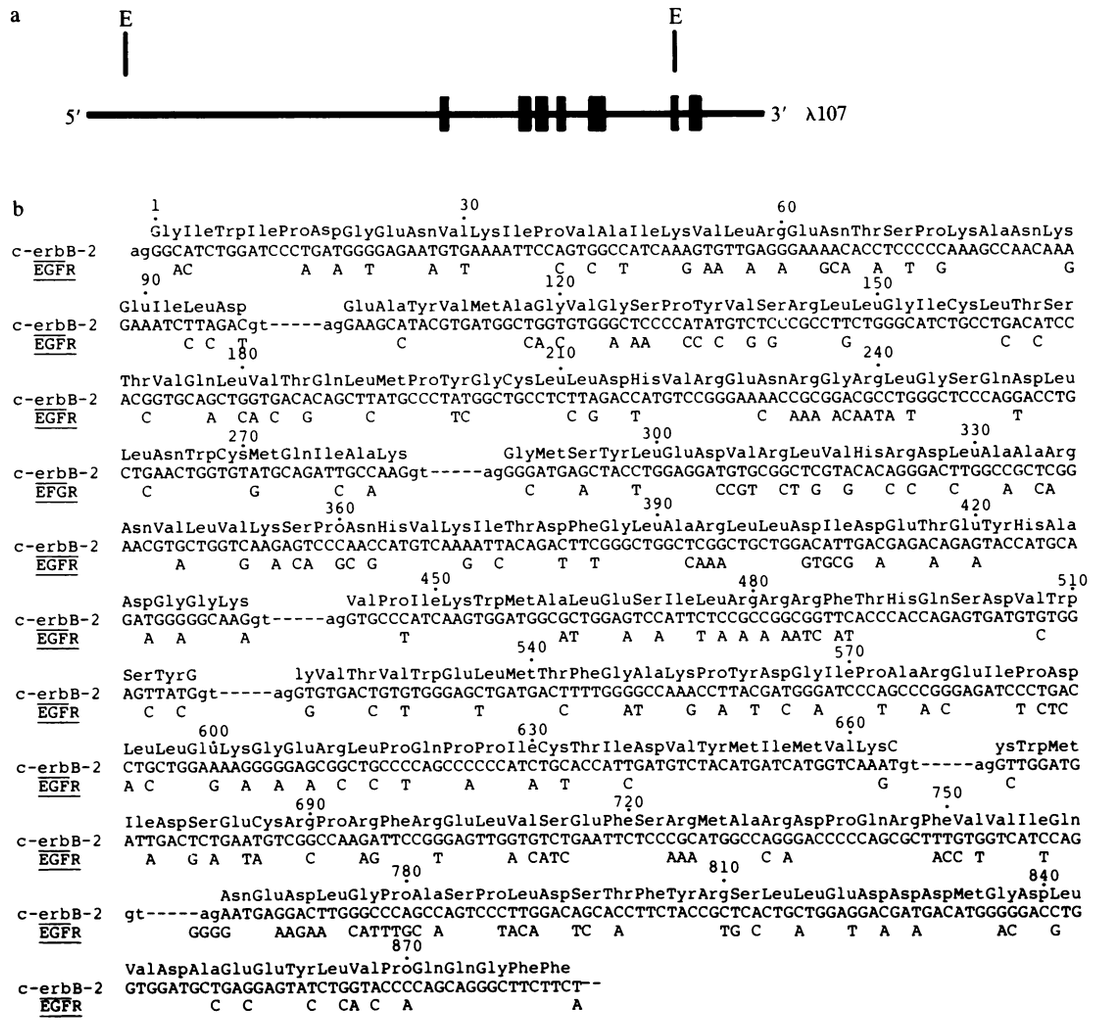

FIG. 3. Nucleotide sequence of the exons for the kinase domain of c-erbB-2. (*a*) Putative exons in the λ107 clone are indicated by thick vertical bars. The two *Eco*RI (E) sites are shown by vertical lines. (*b*) Nucleotide sequence of the putative exons. Restriction fragments that hybridized to v-*erbB* DNA were sequenced. The exons were defined by the splicing consensus sequence and by the high homology of the deduced amino acid sequences to those of the EGF receptor gene *EGFR*. The nucleotide sequence of putative exons of c-*erbB-2* (in capital letters) is compared with that of the EGF receptor gene (8). Only nonidentical nucleotides are shown for the EGF receptor gene. Intron sequences at the splicing sites that flank the exons are shown in lowercase letters. The splicing donor site of the last exon was not identified. The predicted amino acid sequence is shown above the nucleotide sequence.

amplified in A431 cells (data not shown), suggesting that the c-*erbB-1* gene is the same as the EGF receptor gene.

A restriction map of the λ107 DNA insert is shown in Fig. 1*b*. The 440-bp *Kpn* I–*Xba* I fragment (KX fragment) was used as a probe in the following hybridization experiments. This fragment did not contain human repetitive sequences and hybridizes with the v-*erbB* probe under the relaxed conditions. The v-*erbB* probe on the filter represented in Fig. 1*a* was washed off and the filter was rehybridized with the ^{32}P-labeled KX fragment. Fig. 1*c* shows that the KX probe reacted with the restriction fragments (13- and 6.4-kbp *Eco*RI fragments, 6.9-kbp *Sac* I fragment, and 5.1-kbp *Pvu* II fragment) that were not amplified in A431 cells, suggesting that the λ107 insert does not represent the EGF receptor gene. Therefore, we tentatively concluded that the λ107 insert represented a v-*erbB*-related gene (c-*erbB-2*) that differs from the EGF receptor gene.

Then we examined the expression of the v-*erbB*-related genes. RNAs were prepared from human cell lines (K562, MT2, and A431 cells), human embryo fibroblasts, and human placenta. Hybridization was carried out under stringent conditions (Fig. 2 *a* and *b*). Poly(A)$^+$ RNA samples were subjected to blot-hybridization analysis. As reported previously, most transcripts detected with the EGF receptor cDNA probe were of 10 kilobases (kb) and 5.6 kb and were apparently overproduced in A431 cells (Fig. 2*b* and refs. 8 and 23). In addition, variant 2.9-kb mRNA was detected in A431 cells, as reported previously (8, 23). On the other hand, the mRNA that hybridized with the KX probe was a single species of about 4.8 kb (Fig. 2*a*). The level of expression of this mRNA was virtually the same in A431 cells as in placenta and was slightly higher in human embryo fibroblasts than in other cells. Interestingly, transcription of the EGF receptor gene and c-*erbB-2* genes was not detected in the leukemic cell lines K562 and MT2. The above results strongly indicate that the human genome contains two v-*erbB*-related genes, the c-*erbB-1*/EGF receptor gene and the c-*erbB-2* gene.

c-*erbB-2* Encodes a Protein with a Kinase Domain. We determined the nucleotide sequence of the λ107 insert that hybridized with the v-*erbB* DNA and identified seven putative exons flanked by a consensus sequence of splicing junctions (data not shown). Fig. 3 shows that the nucleotide sequences of all seven exons (total, 885 bp) were highly homologous with the corresponding regions of the cDNA clone for the EGF receptor (74%). Only one reading frame deduced from putative exons was not interrupted by termi-

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c-erbB-2  GIWIPDGENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGICLTSTVQL-60
  EGFR     L   E  K        E   A                 S DN H C

c-erbB-2  VTQLMPYGCLLDHVRENRGRLGSQDLLNWCMQIAKGMSYLEDVRLVHRDLAARNVLVKSP-120
  EGFR    I     F     Y   HKDNI   Y     V      N    R              T

c-erbB-2  NHVKITDFGLARLLDIDETEYHADGGKVPIKWMALESILRRRFTHQSDVWSYGVTVWELM-180
  EGFR    Q          K  GAE K    E               H IY

c-erbB-2  TFGAKPYDGIPAREIPDLLEKGERLPQPPICTIDVYMIMVKCWMIDSECRPRFRELVSEF-240
  EGFR      S        S  SSI                                ADS  K    II

c-erbB-2  SRMARDPQRFVVIQ-NEDLGPASPLDSTFYRSLLEDDDMGDLVDAEEYLVPQQGFF
  EGFR     K       YL   GD RMHLP  T  N   A MDEE  D V   D   I
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FIG. 4. Comparison of the deduced amino acid sequences of c-erbB-2 and the EGF receptor (EGFR). The standard one-letter abbreviations are used. Only nonmatching amino acids are shown for the EGF receptor. The kinase domain of EGF receptor is shown as the amino acid sequence between positions 705 and 937 (8). Asterisks indicate residues common to the protein products of the v-*abl*, v-*erbB*, v-*fgr*, v-*fms*, v-*fps*, v-*ros*, v-*src*, and v-*yes* genes.

nation codons. The amino acid sequence deduced from this open-reading frame suggested that these exons in c-*erbB-2* could encode a polypeptide characteristic of the kinase domain (residues 1–233 in Fig. 4) that showed high homology with the EGF receptor kinase domain (82%). The amino acid sequence of the kinase domain of the c-*erbB-2* product is highly homologous to that of the v-*erbB* product and distantly related to those of the protein products of other members of the *src* gene family (Table 1).

Conservation of the c-*erbB-2* Gene in Vertebrates. High molecular weight DNAs were prepared from chicken blood, mouse FM3A cells, and rat spleen. The DNAs were digested with *Eco*RI and subjected to Southern hybridization with the ^{32}P-labeled KX probe. Since the hybridization conditions were stringent enough to avoid detection of the EGF receptor gene with the KX probe (see Fig. 5*a*, lane 1), all the fragments shown in Fig. 5 are specific to the c-*erbB-2* gene. Thus, we conclude that c-*erbB-2* is conserved in vertebrates.

The possible relation between c-*erbB-2* and the recently discovered *neu* oncogene was examined by Southern blot hybridization. The ^{32}P-labeled KX probe hybridized with a rat *Eco*RI fragment of more than 23 kbp and with a 4.4-kbp fragment generated by digestions of the DNA with *Eco*RI and *Bam*HI (Fig. 5). These fragments are the same sizes as the respective restriction fragments of the *neu* oncogene (24).

Association of Amplification of the c-*erbB-2* Gene with a Primary Human Tumor. Next we examined whether the c-*erbB-2* gene is amplified in human cancers. High molecular weight DNAs were isolated from human placenta, A431 cells, and several primary tumors (one neuroblastoma, two epidermoid cell carcinomas, and one adenocarcinoma of the salivary gland). These DNAs were digested with *Eco*RI and analyzed by Southern blot hybridization with the KX probe. Two *Eco*RI fragments (13 and 6.4 kbp) were identified in all the DNA samples and were amplified about 30-fold in the adenocarcinoma of the salivary gland (UY adenocarcinoma), indicating possible involvement of c-*erbB-2* gene expression in this cancer (Fig. 6*a*). We do not know whether the entire c-*erbB-2* gene is amplified in this tumor, since the KX probe detects only part of the c-*erbB-2* gene. When the same filters were hybridized with the EGF receptor cDNA probe, amplification of the EGF receptor gene was observed in A431 DNA, as has been reported (8, 23), but not in the UY adenocarcinoma. Owing to the limited amount of tissue available, RNA of the UY adenocarcinoma could not be analyzed. We have detected amplification and enhanced expression of the c-*erbB-2* gene in a cell line (MKN-7) established from human gastric cancer (data not shown).

DISCUSSION

There are two v-*erbB*-related genes in the human genome: the c-*erbB-1*/EGF receptor gene and the c-*erbB-2* gene. The c-*erbB-2* gene is apparently not a pseudogene of the EGF receptor gene because it consists of both exons and introns and is transcribed in a cell-type-specific manner. We could not find any termination codon interrupting the reading frame of the putative kinase domain coded by c-*erbB-2*. In addition, we have shown that the c-*erbB-2* gene is conserved in chickens, mice, rats, and humans, indicating that this gene is not confined to the human genome and probably fulfills an indispensable function. Obviously, efforts to identify a protein product of the c-*erbB-2* gene are required.

The amino acid sequence deduced from the nucleotide sequence of c-*erbB-2* exons in λ107 suggests that the c-*erbB-2* gene may code for a tyrosine-specific protein kinase. The putative kinase domain encoded by the c-*erbB-2* gene shows 82% homology with that of the EGF receptor and the v-erbB protein. The eight protein products of the *src* family show tyrosine kinase activity *in vitro*. All the amino acid residues that are common to these eight proteins are also conserved in the c-*erbB-2* gene product, strongly supporting the idea that this gene could encode a protein kinase. Our nucleotide sequence data further show that the c-erbB-2 protein carries an amino acid sequence (residues 234–295) that is 65% homologous to the corresponding portion of the EGF receptor. Since the deduced amino acid sequence did not contain a putative transmembrane portion, it is not known whether c-*erbB-2* encodes a receptor-like protein or only a polypeptide similar to the intracellular portion of the EGF receptor. Analysis of the amino-terminal portion of the protein is necessary to determine whether it has a transmembrane sequence and a domain that recognizes some growth factor(s).

Recently, the *neu* oncogene was identified, by gene-transfer techniques, in the genomes of four neuro/glioblastoma cell lines derived from chemically induced rat tumors (24). The *neu* gene is related to the v-*erbB* gene and encodes a tumor antigen with a molecular weight of 185,000 (p185). p185 is serologically related to, but distinct from, the EGF receptor (25). Our data show that the rat c-*erbB-2* gene yields restriction fragments of the same sizes as does the rat *neu* oncogene when digested with *Eco*RI or *Eco*RI plus *Bam*HI.

Table 1. Amino acid sequence homology between the c-*erbB-2*-encoded kinase domain and those encoded by various viral oncogenes

Viral oncogene:	*erbB*	*src*	*abl*	*yes*	*fgr*	*ros*	*fps*	*mil*	*fms*	*mos/rel*
% homology:	82	43	42	42	41	38	37	28	27	<25

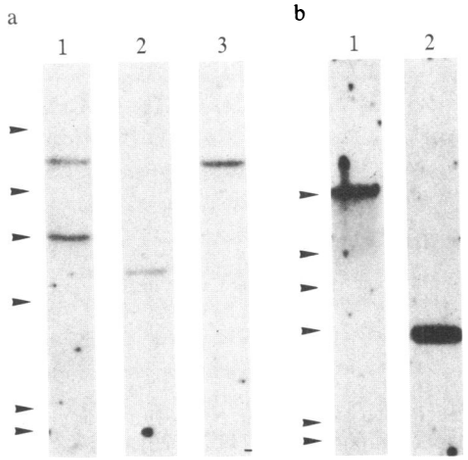

FIG. 5. Conservation of the c-*erbB-2* gene in vertebrates. (*a*) Conserved c-*erbB-2* sequences in chicken and mouse. Nitrocellulose filters containing *Eco*RI digests of DNA from human placenta (lane 1), mouse FM3A cells (lane 2), and chicken blood (lane 3) were probed with the KX DNA. (*b*) Close similarity of the c-*erbB-2* gene to the *neu* oncogene. Rat DNA was cleaved with either *Eco*RI (lane 1) or *Eco*RI plus *Bam*HI (lane 2). The digests were analyzed by Southern blot hybridization with the KX probe. Arrowheads indicate positions of fragments from *Hin*dIII digestion of bacteriophage λ DNA (see Fig. 1*a*).

These findings lead us to speculate that there is a close relation between c-*erbB-2* and *neu*.

Amplification of protooncogenes such as *myc* and *ras* has been reported to be associated with various human cancers (26–30). This amplification suggests that the protein products of the amplified protooncogenes are involved in some stage of tumorigenesis. Of particular interest is the role(s) of growth factor receptors in tumorigenesis, which is inferred from the observations that EGF receptors are expressed in large numbers on the surface of cells of epidermoid carcinomas (31–33). Since the c-*erbB-2* gene could code for a protein similar to the EGF receptor or at least its intracellular domain and was amplified in an adenocarcinoma of the salivary gland, the gene products expressed abnormally in this tumor

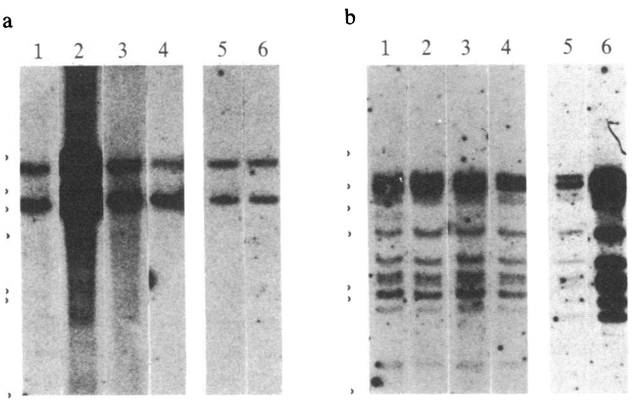

FIG. 6. Amplification of the c-*erbB-2* gene in human primary tumors. (*a*) Nitrocellulose filters containing *Eco*RI digests of tumor DNAs were probed with c-*erbB-2*-specific KX DNA. (*b*) After removal of the first probe, the filters were then hybridized with the EGF receptor cDNA probe. Hybridization was carried out under stringent conditions. DNAs were isolated from a neuroblastoma (lane 1), an adenocarcinoma of the salivary gland (lane 2), two squamous cell carcinomas (lanes 3 and 4), placenta (lane 5), and the A431 cell line (lane 6). Markers at left in *a* and *b* indicate positions of fragments from *Hin*dIII digestion of λ DNA.

might function in establishing and maintaining the cancerous state.

Note Added in Proof. King *et al.* (34) have independently isolated an *erbB-2* gene amplified in a human mammary carcinoma.

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