3000 Ci/m*M*) (UTP) was then added, and the nuclear suspension was incubated at 30°C for 30 minutes, after which time 15 μl of DNase I (5 μg/ml) in 10 m*M* $CaCl_2$ (5 μg/ml) was added. After 5 minutes at 30°C, the reaction was made 1× SET (1 percent sodium dodecyl sulfate (SDS), 5 m*M* EDTA, 10 m*M* tris-HCl, *p*H 7.4), and proteinase K was added to a concentration of 200 μg/ml. After incubation at 37°C for 45 minutes, the solution was extracted with an equal volume of a mixture of phenol and chloroform, and the interphase was again extracted with 100 μl of 1× SET. Ammonium acetate (10*M*) was added to the combined aqueous phases (original plus reextraction) to a final concentration of 2.3*M*, an equal volume of isopropyl alcohol was added, and nucleic acid was precipitated (−70°C for 15 minutes). The precipitate was centrifuged in a microcentrifuge for 10 minutes, and the pellet was resuspended in 100 μl of TE (10 m*M* tris-HCl, 1 m*M* EDTA) and centrifuged through a G-50 (medium) spin column. The eluate was made 0.2*M* in NaOH and after 10 minutes on ice, HEPES was added to a concentration of 0.24*M*. Two and one-half volumes of ethanol were then added, and the solution containing the precipitate held overnight at −20°C. After centrifugation in a microcentrifuge for 5 minutes, the pellet was resuspended in hybridization buffer, which consisted of [10 m*M* TES, *p*H 7.4, 0.2 percent SDS, 10 m*M* EDTA, 0.3*M* NaCl, 1× Denhardt's, and *Escherichia coli* RNA (250 μg/ml)]. Nitrocellulose filters containing plasmid DNA's were prepared with a Schleicher & Schuell Slot Blot Apparatus under conditions suggested by S and S, except that wells were washed with 10× SSC (saline sodium citrate). These filters were first hybridized in the hybridization solution described above for a minimum of 2 hours at 65°C. After this preliminary hybridization, the filters were hybridized to the runoff products in hybridization solution for 36 hours. A typical reaction contained 2 ml of hybridization solution with 1 × 10^7 cpm/ml. After hybridization, filters were washed for 1 hour in 2× SSC at 65°C. The filters were then incubated at 37°C in 2× SSC with RNase A (10 mg/ml) for 30 minutes and were subsequently washed in 2× SSC at 37°C for 1 hour. Alternatively, after hybridization the filters were washed twice for 15 minutes in 0.1 percent SDS, 2× SSC at room temperature, and then washed at 60°C (0.1 percent SDS, 0.1× SSC) for 30 minutes. Either protocol for processing of the filters after hybridization yielded the same specificity in signal. Filters were then exposed to Kodak XAR film in cassettes containing Lightening-Plus screens at −70°C for various times.

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RESEARCH ARTICLE

Tyrosine Kinase Receptor with Extensive Homology to EGF Receptor Shares Chromosomal Location with *neu* Oncogene

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Growth factors and their receptors are involved in the regulation of cell proliferation, and several recent findings suggest that they also play a key role in oncogenesis (*1–4*). Of approximately 20 identified oncogenes, the three that have been correlated with known cellular proteins are each related to either a growth factor or a growth factor receptor. The B chain of platelet-derived growth factor (PDGF) is encoded by the proto-oncogene c-*sis* (*2*), the erb-B oncogene product gp68 is a truncated form of the epidermal growth factor (EGF) receptor (*3*), and the proto-oncogene c-*fms* may be related or identical to the receptor for macrophage colony-stimulating factor ($CSF\text{-}1^R$) (*4*).

The receptor-related oncogenes are members of a gene family in that each has tyrosine-specific protein kinase activity, and is associated with the plasma membrane (*5*). Such features are also shared by several other polypeptide hormones, including insulin (*6*), PDGF (*7*), and insulin-like growth factor 1 (IGF-1) (*8*); hence more connections may be found between tyrosine kinase growth factor receptors and tyrosine kinase oncogene products.

Comparison of the complete primary structure of the human EGF receptor (*9*) with the sequence of the avian erythroblastosis virus (AEV) transforming gene, v-erbB (*10*), revealed close sequence similarity; in addition, there were amino and carboxyl terminal deletions that may reflect key structural changes in the generation of an oncogene from the gene for a normal growth factor receptor (*3, 9*). Another oncogene, termed *neu*, is also related to v-erbB and was originally identified by its activation in ethylnitrosourea-induced rat neuroblastomas (*11*). In contrast to v-erbB, which encodes a 68,000-dalton truncated EGF receptor, the *neu* oncogene product is a 185,000-dalton cell surface antigen that can be detected by cross-reaction with polyclonal antibodies against EGF receptor (*11*); *neu* may itself be a structurally altered cell surface receptor with homology to the EGF receptor and binding specificity for an unidentified ligand.

Using v-erbB as a screening probe, we isolated genomic and cDNA clones coding for an EGF receptor-related, but distinct, 138,000-dalton polypeptide having all the structural features of a cell surface receptor molecule. On the basis of its structural homology, this putative receptor is a new member of the tyrosine-specific protein kinase family. It is encoded by a 4.8-kb messenger RNA (mRNA) that is widely expressed in normal and malignant tissues. We have localized the gene for this protein to q21 of chromosome 17, which is distinct from the EGF receptor locus, but coincident with the *neu* oncogene mapping position (*12*). We therefore consider the possibility that we have isolated and characterized the normal human counterpart of the rat *neu* oncogene.

Tyrosine kinase–type receptor gene and complementary DNA. As part of our attempts to isolate and characterize the chromosomal gene coding for the human cellular homologue of the viral erbB gp68 polypeptide, AEV-ES4 erbB sequences (2.5-kb Pvu II fragment of pAEV) (*13*) were used as a ^{32}P-labeled hybridization probe for the screening of a human genomic DNA library at reduced stringency

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(*14*). Clone λc-erbB/1 was isolated; it contained a hybridizing 1.8-kb Bam HI fragment, which was subjected to DNA sequence analysis. The 1838-bp sequence contains three complete and one partial erbB-homologous exons separated by short intervening sequences (Fig. 1). Comparison of this human gene sequence with our complete cDNA-derived human EGF receptor protein sequence (*9*) revealed 32 differences (18.7 percent) within the 171 amino acid stretch of combined exons, suggesting that this gene fragment was not derived from the human EGF receptor gene. Since this gene may code for an unknown tyrosine kinase–type receptor that is closely related to the human EGF receptor, we named it HER2.

Northern blot analysis (*15*) with the ^{32}P-labeled 1.8-kb HER2 fragment as a hybridization probe revealed a 4.8-kb mRNA in human term placenta poly(A)$^+$ RNA, distinct from the 5.8- and 10.5-kb EGF receptor mRNA's also present at high levels in this tissue (Fig. 2a, lane 1). Thus, we had isolated a portion of an EGF receptor–erbB-related but distinct gene. To obtain its complete primary structure, two single-stranded synthetic oligonucleotide probes (*16*) were prepared from HER2 exon sequence regions that differed sufficiently (less than 60 percent nucleotide sequence homology) from EGF receptor DNA sequences (Fig. 1, 1 and 2) and used to screen a term placenta complementary DNA (cDNA) library of 2×10^6 independent recombinant clones in λgt10 (*17*). Fifty-two clones were isolated; they hybridized strongly with both synthetic probes and weakly with an EGF receptor cDNA fragment (HER64-3) (*9*) containing the homologous region within the tyrosine kinase domain. One of these, λHER2-436, had the longest cDNA insert (4.5 kb), consisting of three Eco RI fragments (1.4, 1.5, and 1.6 kb).

The complete cDNA sequence of this clone is shown in Fig. 3. The longest open reading frame starting with a methionine codon codes for a 1255 amino acid polypeptide (137,828 daltons) and contains the 171 residues encoded by the four exons in the 1.8-kp HER2 gene Bam HI fragment (Fig. 1). This 3765-bp coding sequence is flanked by 150 bp of 5′ untranslated sequence and a TGA stop codon, followed by a 627-nucleotide 3′ untranslated sequence. No stop codon is found in the 5′ untranslated region. In support of our assignment, however, the initiation codon at position 151 is flanked by sequences that follow perfectly Kozak's rule (*18*) for translation initiation

potential poly(A) addition signal sequence (AATATA) 12 nucleotides upstream from a stretch of 15 adenylate residues. We are not certain if this $(A)_{15}$ stretch is part of a poly(A) tail or represents an internal poly(A) stretch of a longer 3′ untranslated sequence.

Abstract. *A novel potential cell surface receptor of the tyrosine kinase gene family has been identified and characterized by molecular cloning. Its primary sequence is very similar to that of the human epidermal growth factor receptor and the v-erbB oncogene product; the chromosomal location of the gene for this protein is coincident with the* neu *oncogene, which suggests that the two genes may be identical.*

Comparison of EGF receptor and HER2 sequence. As already indicated by the v-erbB sequence homology used to isolate HER2, the putative HER2 protein is very similar in its overall domain organization and sequence to the EGF receptor. Nevertheless, there are differences that are likely to define a specific biological role for the HER2 polypeptide.

The predicted HER2 polypeptide contains each of the domain features found in hormone receptor precursors, such as those for EGF and insulin (*9*, *19*). Such features are apparent in the hydropathy profile (*20*) comparison (Fig. 4a). On the basis of this comparison, and on amino acid sequence alignment with the EGF receptor (Fig. 4b, region 1), we predict a 21 amino acid signal sequence (Fig. 4b, 1), an amino terminal serine residue, and a 632 amino acid putative extracellular ligand-binding domain; a highly hydrophobic, 22–amino acid transmembrane anchor domain separates the extracellular domain from a 580-residue-long carboxyl-terminal cytoplasmic domain, which possesses the highest homology to v-erbB and other members of the tyrosine kinase family.

The 632–amino acid, putative HER2 ligand binding domain is about 40 percent homologous with the 621-residue

Fig. 1. Partial sequence of the HER2 gene. A partial Hae III-Alu I genomic library (*14*) of human fetal DNA in λ Charon 4A was screened using a radiolabeled 2.5-kb Pvu II fragment of pAEV (*13*) containing coding sequences for the tyrosine kinase domain. Hybridization was as described elsewhere (*31*), except that 30 percent formamide was used at 42°C. Three independent clones were isolated which shared a 1.8-kb hybridizing Bam HI fragment. This fragment and subsets thereof were isolated, subcloned into M13mp10 and M13mp11, and sequenced (*32*). The intron-exon organization was determined by comparison with v-erbB sequences (*10*). Amino acid numbering is based on the complete cDNA sequence shown in Fig. 3. Nucleotide sequence differences with the human EGF receptor sequence are shown in the regions that were used for the design of synthetic oligonucleotide probes 1 (30 nucleotides) and

extracellular EGF binding domain of the EGF receptor. This homology includes two cysteine-rich subdomains of 26 and 21 regularly organized cysteine residues (Figs. 4a and 2c, subdomains 2 and 3), all of which are conserved in the EGF receptor. The cysteine residue spacing in this region is also homologous with the single cysteine-rich domain in the insulin receptor α subunit (*19*). In contrast, HER2 contains only eight potential *N*-linked glycosylation target sites (Asn-X-Thr or Ser) as compared to 12 in the corresponding region of the EGF receptor. Only five of these are conserved with respect to their relative position in each polypeptide.

The hydrophobic, putative membrane anchor sequence located between residues 653 and 676 (Fig. 4b, region 4) is flanked at its carboxyl terminus by a stretch of amino acids of predominantly basic character (KRRQQKIRKYTMRR) (*21*), as is found in the EGF receptor sequence (*9*) (Fig. 4b, region 5). This region of the EGF receptor contains Thr^{654}, which plays a key role in protein kinase C–mediated receptor modulation (*22*). A homologous threonine residue is embedded in a basic environment in the HER2 sequence at position 685 (Fig. 4, a and b).

The region of most extensive homology (78.4 percent) between EGF receptor and HER2 (beginning at residue 687) extends over 343 amino acids and includes sequences specifying the adenosine triphosphate (ATP) binding domain (*23*) and tyrosine kinase activity (Fig. 4b, region 6) (*5*). This region is also the most conserved between v-erbB and EGF receptor (95 percent) (*9*). The collinear homology between the EGF receptor-erbB and HER2 ceases at position 1032, but introduction of gaps into the EGF receptor or HER2 sequences reveals continued, although decreased, relatedness (Fig. 4b, region 7). This sequence alignment suggests that the two genes evolved by duplication of an ancestral receptor gene, and that subsequent nucleotide sequence divergence in this carboxyl terminal domain led to diverged biological roles for the encoded polypeptides.

The carboxyl terminal domain of HER2 is characterized by an unusually high proline content (18 percent) and predominant hydrophilicity (Fig. 4a). These general features are also found in the EGF receptor carboxyl terminal domain with a 10 percent proline content. The sequences in this region that are found to be conserved are almost exclu-

and two minor (Tyr^{1148}, Tyr^{1068}) in vitro autophosphorylation sites in the human EGF receptor (*24*) (Fig. 4, a and b). Three of these tyrosine residues of HER2 (positions 1139, 1196, 1248) are flanked by homologous sequences PQPEYV, ENPEYL, and ENPEYL (*21*), respectively (Fig. 4b, region 7).

HER2 chromosomal location. In situ hybridization of two ^{3}H-labeled HER2 probes (legend, Fig. 5a) to human chromosomes resulted in specific labeling at bands q12→q22 of chromosome 17 (Fig. 5a). Metaphase cells (100) were analyzed for each probe; 40 percent of cells scored for HER2 probe 1 (HER2-1) had silver grains over 17q12→q22 (Fig. 5b). Of the 209 grains observed, 42 (20 percent) were found at this specific region, with no other site labeled above background. For HER2 probe 2, 36 percent of cells had silver grains over the q12→q22 bands of chromosome 17. Of all silver grains, 17 percent (42/246) were localized to this chromosomal region. A secondary site of hybridization with 3.3 percent (8/246) of silver grains was detected at bands p13→q11.2 of chromosome 7.

To test whether this secondary site represented cross-hybridization with the EGF receptor gene, in situ hybridization was carried out with ^{3}H-labeled EGF

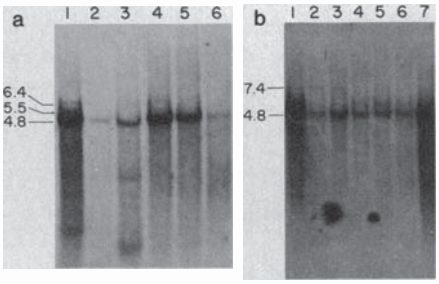


Fig. 2. Northern blot hybridization analysis of normal and malignant human tissues. (a) Fetal tissues; (lane 1) term placenta, (lane 2) 20-week placenta, (lane 3) 20-week liver, (lane 4) 20-week kidney, (lane 5) 20-week lung, (lane 6) 20-week brain. (b) Embryonic tumors; (lane 1) hepatoblastoma, (lanes 2 and 3) Ewing sarcoma, (lane 4) rhabdomyosarcoma, (lanes 5 and 6) neuroblastoma, (lane 7) Wilms' tumor. Total poly(A)$^+$ RNA was isolated as described (*33*); 4 μg per lane was analyzed on a 1 percent formaldehyde-agarose gel. ^{32}P-Labeled HER2-1 and HER-2 (legend to Fig. 5) were used as hybridization probes under high stringency conditions [50 percent formamide, 5× Denhardt's solution, 5× standard saline citrate (SSC), sonicated salmon sperm DNA (50 μg/ml), 50 μ*M* sodium phosphate buffer (*p*H 6.8), 1 m*M* sodium pyrophosphate, and 10 μ*M* ATP at 42°C for 16 hours; filters were washed three times for 15 minutes at 45°C with 0.2× SSC]. The filters were exposed at −60°C with a Cronex Lightning Plus intensifying screen (Dupont) for 7 days. Rat ribosomal RNA's were used as size standards

receptor subclone 64-3. Of 100 cells examined, 30 had silver grains at bands p13→q11.2 of chromosome 7 and 3 percent (5/166) of total grains were found over q12→q22 of chromosome 17. With the other variant probe (HER2-1) no grain accumulation was observed at the EGF receptor site on chromosome 7.

Southern blot analysis (*25*) of DNA extracted from nine somatic cell hybrids from human and rodent cells confirmed the localization of HER2 sequences to chromosome 17. ^{32}P-labeled HER2-1 and HER2-2 probes were hybridized to the same set of Eco RI–digested DNA samples. With HER2-1, a 13-kb hybridizing band was detected in human DNA (Fig. 5c, lane 1) and in DNA samples from hybrids containing human chromosome 17 (Fig. 5c, lanes 6, 8, 10, and 12). Likewise, hybridization of HER2-2 to a 6.6-kb DNA fragment was observed in human control DNA (Fig. 5c, lane 1) and in hybrids containing human chromosome 17 (Fig. 5c, lanes 6, 8, 10, and 12). Chromosome 17 was the only chromosome with perfect concordant segregation; all other chromosomes were excluded by two or more discordant hybrids.

Regional localization to chromosome 17 was also confirmed by Southern blot analysis. In a mouse-human hybrid containing a rearranged human chromosome 17 with region 17q21→qter, the human HER2 restriction fragments were detected (Fig. 5c, lane 4). The HER2 gene was therefore localized to region 17q21→qter, in agreement with the localization made by in situ hybridization.

Even though a low level of hybridization with probe HER2-2 was seen at the site of the EGF receptor gene on chromosome 7, we were able to show that this finding represented cross-hybridization. In a control experiment an EGF receptor probe cross-hybridized to the same extent with the HER2 site on 17q.

Taken together, the results of the in situ and Southern blot hybridizations permit the site of the HER2 sequences to be further narrowed down to bands 17q21-q22, with the major peak of silver grains at band 17q21.

HER2 expression in normal and malignant tissues. To obtain further clues regarding the function of this receptor both in normal cells and in neoplasms, Northern hybridization analyses (*15*) were carried out with several normal human tissues and randomly collected tumors. A hybridizing 4.8-kb mRNA was detected in all human fetal tissues analyzed, in-

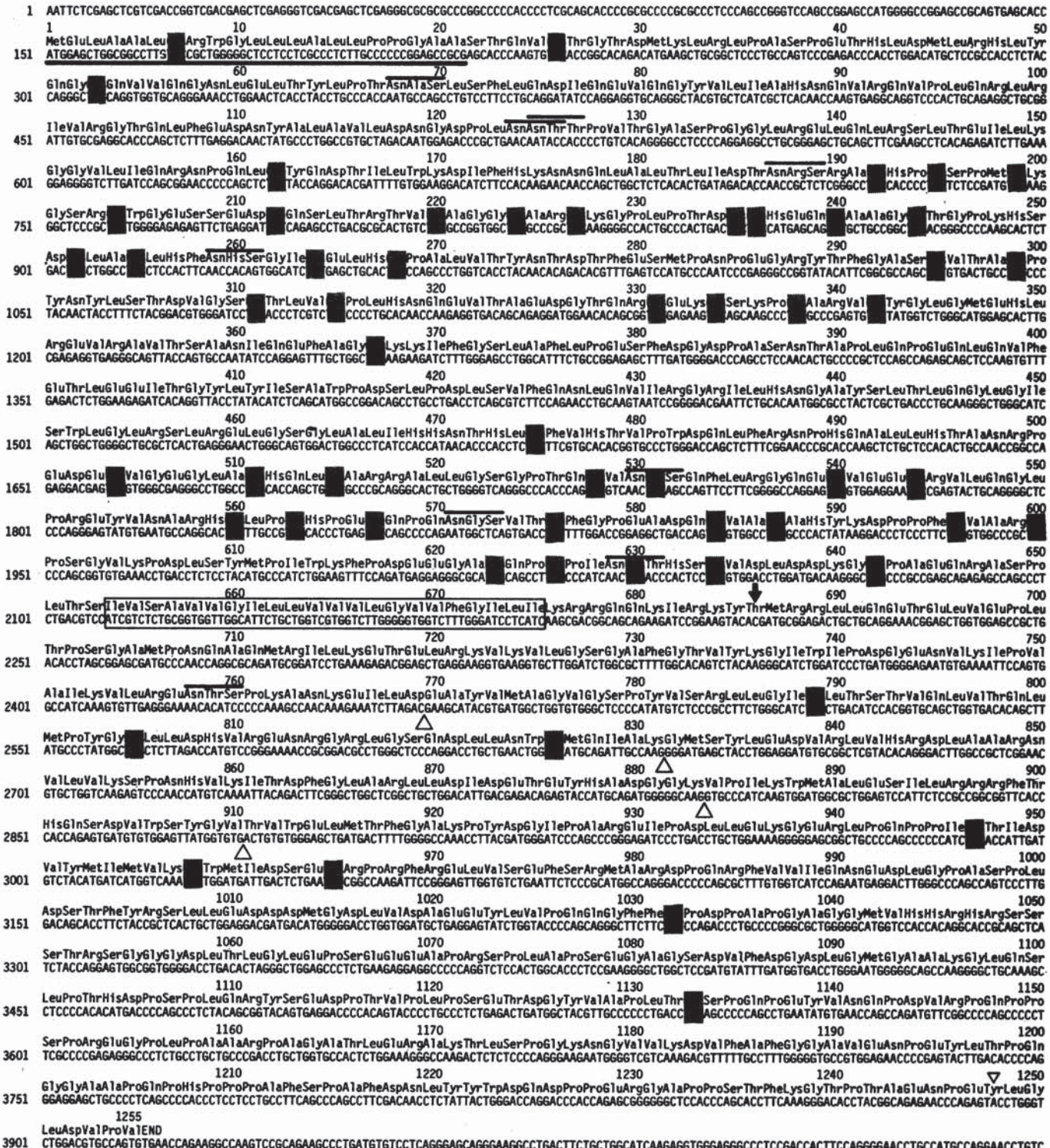
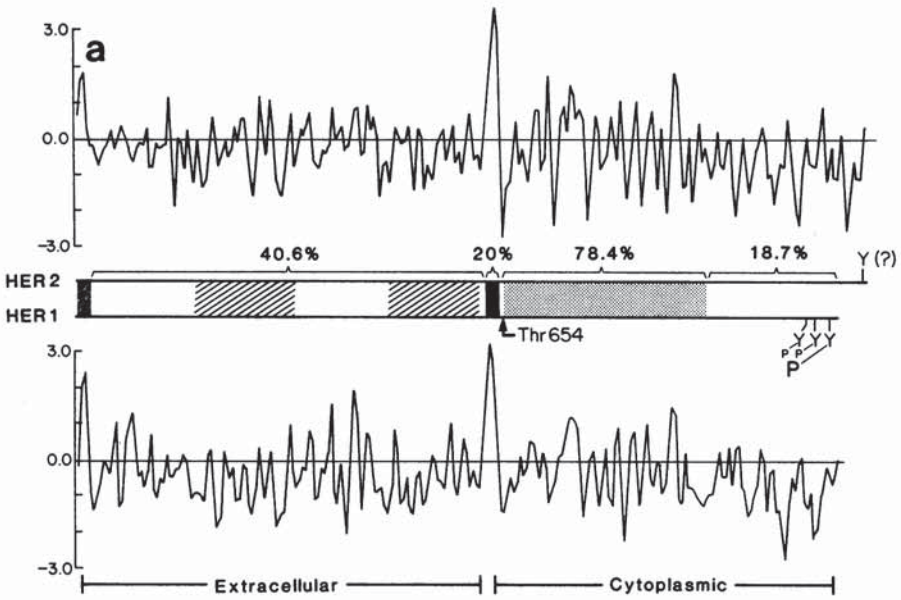


Fig. 3. Complete nucleotide and amino acid sequences of HER2 (clone λHER2-436). Synthetic probes 1 and 2 (Fig. 1) were used to screen 2 × 10^6 clones of a human placental cDNA library in λgt10 (*17*) as described (*9*). Fifty-two clones were isolated and characterized by Eco RI restriction digestion and Southern blot hybridization (*25*) with the radiolabeled synthetic oligonucleotides (*34*) and, on a duplicate blot, an EGF receptor Eco RI fragment (HER64-3) as probes. Labeling of oligonucleotides was achieved with 1 unit of T4 polynucleotide kinase (New England Biolabs), 10 pmol of oligonucleotide and 30 pmol of [γ-^{32}P]ATP (Amersham) as specified by the supplier. HER64-3 was labeled to high specific activity (>10^8 cpm/μg) as described (*34*). Clone λHER2-436 was used for nucleotide sequence analysis (*32*) of overlapping subclones. Amino acids are numbered starting with the initiation methionine (1). Underlining indicates the putative signal sequence (heavy) and the potential poly(A) addition signal (fine). Lines on top of the sequence indicate potential glycosylation sites, the black arrow demarcates the EGF receptor threonine 654 analogue (Thr 686), shading indicates cysteine residues, boxing shows the putative transmembrane region, large open triangles indicate some locations of introns in the HER2 gene, and the small triangle emphasizes a possible tyrosine autophosphorylation site by homology to Tyr 1173 in the EGF receptor sequence.

from a single fetus (Fig. 2a). Two mRNA's, of 5.4 and 6.4 kb, were also detected in term placenta. No cross-hybridization with the 5.8-kb and 10.5-kb EGF receptor mRNA's in term placenta mRNA was observed under these stringent hyridization conditions (legend, Fig. 2). Normal adult human tissues, including kidney, liver, skin, lung, jejunum, uterus, stomach, and colon, contained lower but significant amounts of the same 4.8-kb mRNA. Because of the magnitude of fetal expression, we also examined several embryonic tumors (Fig. 2b); each expressed large amounts of the 4.8-kb transcript, although not more than that detected in normal fetal tissue.

Thus, it appears that the HER2 gene is widely expressed, in both normal adult tissues and in several normal fetal tissues. While detected in most embryonic tumors, the HER2 gene was not present at higher levels than in fetal tissues; thus, the particular level may reflect the state of differentiation of a given tumor.

HER2 structurally characterized as cell surface receptor. Using the transforming gene of the avian erythroblastosis virus, v-erbB, as a hybridization probe, we isolated genomic and cDNA sequences of an uncharacterized human gene. The 1255 amino acid polypeptide sequence



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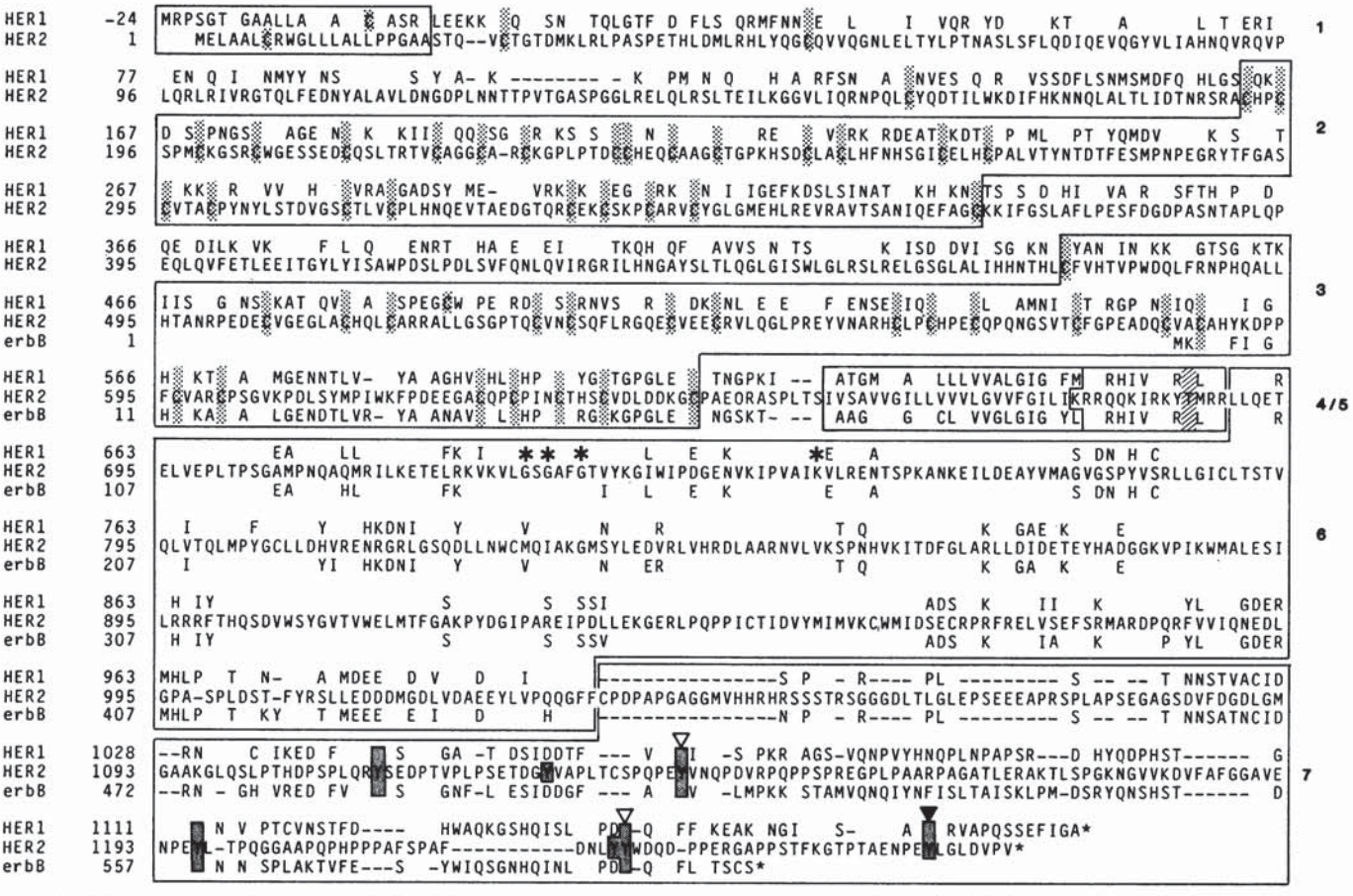

Fig. 4. (a) Hydropathy analysis (*20*) of HER2 (1255 amino acids) coding sequences and comparison with EGF receptor (HER1; 1210 amino acids). Different receptor domains and the extent of amino acid sequence homology are indicated. The autophosphorylation tyrosines in the EGF receptor sequence are shown, as is a potential analogue in HER2. The signal sequence is shown by fine shading, the cysteine-rich subdomains by hatching, the transmembrane region by a black bar, and the tyrosine kinase domain by coarse shading. (b) Comparison of HER2, EGF receptor (HER1), and v-erbB amino acid sequences. Identical residues are deleted, gaps are introduced to optimize alignment, cysteine residues are shaded, and carboxyl terminal tyrosines are in shaded boxes. The black and open triangles indicate the positions of the major and minor autophosphorylation sites in the EGF receptor, respectively. Asterisks indicate residues involved in ATP binding. Boxed regions include (i),

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