

AD _____

GRANT NUMBER DAMD17-94-J-4179

TITLE: Her-2/Neu and Breast Cancer

PRINCIPAL INVESTIGATOR: Joni Doherty

CONTRACTING ORGANIZATION: Oregon Health Sciences University
Portland, Oregon 97201-3042

REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 2

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY <i>(Leave blank)</i>	2. REPORT DATE August 1998	3. REPORT TYPE AND DATES COVERED Annual (8 Jul 97 - 7 Jul 98)
4. TITLE AND SUBTITLE Her-2/Neu and Breast Cancer		5. FUNDING NUMBERS DAMD17-94-J-4179
6. AUTHOR(S) Joni Doherty		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Oregon Health Sciences University Portland, Oregon 97201-3042		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES		19990209 124
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		12b. DISTRIBUTION CODE
13. ABSTRACT <i>(Maximum 200 words)</i> The HER-2/ <i>neu</i> proto-oncogene is developmentally expressed as a 185 kDa trans-membrane tyrosine kinase. Overexpression of HER-2 occurs in up to 30% of human adenocarcinomas and has been most extensively studied in breast and ovarian cancers, where it predicts poor prognosis for survival of the patient. Although HER-2 gene amplification is a common mechanism for overexpression, many human tumors and cell lines display HER-2 mRNA and protein overexpression in excess of the level accountable by gene copy number, or in absence of gene amplification. Moreover, expression of HER-2 can be upregulated without concomitant gene expansion, indicating mechanisms for transcriptional and post-transcriptional upregulation. Such mechanisms require further investigation. The model cell line SK-OV-3 exhibits the highest HER-2 protein levels and evidence for one or more of these upregulation mechanisms in addition to gene amplification. Furthermore, in excess overexpression of the normal-sized 4.5 kb HER-2 mRNA transcript, SK-OV-3 also expresses an abundant alternative HER-2/ <i>neu</i> transcript of 8 kb, which has not yet been characterized. Preliminary data suggests that this aberrant 8 kb transcript is translated into p185HER-2, has unaltered 5'UTR and coding sequences, yet the 8 kb transcript displays increased mRNA stability and has an extended 3'UTR sequence. The present work focuses on further characterizing this transcript in terms of (1) primary sequence and secondary structure, (2) role in HER-2/ <i>neu</i> overexpression, (3) and developmental expression.		
14. SUBJECT TERMS Breast Cancer HER-2/<i>neu</i>, proto-oncogene, cDNA, 3'UTR, 5'UTR, RT-PCR, RACE, p185^{HER2/<i>neu</i>}		15. NUMBER OF PAGES 24
		16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified
20. LIMITATION OF ABSTRACT Unlimited		

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

JD ✓ Where copyrighted material is quoted, permission has been obtained to use such material.

JD ✓ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

JD ✓ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.


NA In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

JD ✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

JD ✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

JD ✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

JD ✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

 8-1-98

PI - Signature Date

Table of Contents

Front Cover.....	1
Standard Form (SF) 298, Report Documentation Page.....	2
Foreword.....	3
Table of Contents.....	4
Introduction.....	5
Body.....	7
Conclusions.....	12
Future Work.....	13
References.....	15
Appendix A.....	17
Appendix B.....	23

Characterization of an Alternative HER-2/*neu* mRNA Transcript

INTRODUCTION

Nature of the Problem and Background of Previous Work

The HER-2/*neu* (*c-erbB-2*) is developmentally expressed in many tissues as a 185 kDa transmembrane tyrosine kinase (p185HER-2/*neu*) with extensive homology to the Epidermal Growth Factor Receptor (EGFR) (1). p185HER-2/*neu* has been shown to signal through the mitogen-activated protein (MAP) kinase pathway, inducing nuclear events that may lead to proliferation and growth of tumor cells (2). Like EGFR, overexpression of HER-2/*neu* has been reported in an array of human carcinomas (3), most notably in up to 30% of breast and ovarian cancers. Moreover, HER-2/*neu* overexpression is associated with increased tumor invasiveness, malignancy, and predicts poor prognosis for survival of the patient (4).

The mechanisms for p185HER-2/*neu* overexpression have not been entirely elucidated, although HER-2/*neu* gene amplification has been shown (3, 5, 7). However, many tumors and cell lines display overexpressed HER-2/*neu* mRNA and protein, in absence of gene amplification (3, 7, 8). Although mechanisms for gene amplification and transcriptional upregulation are not well understood, studies suggest selection for p185HER-2/*neu* overexpression in some human breast and ovarian cancers and cell lines (3, 7, 8). In fact, HER-2 transcriptional upregulation, demonstrated to occur in the breast carcinoma cell line BT474, as well post-transcriptional upregulation, in SKOV-3 (18), have been implicated.

Mechanisms for overexpression of p185HER-2/*neu* involving sequence variations in the HER-2 gene and within its mRNA transcript require further investigation. Possible mechanisms for overexpression include: (a) increased gene copy number, (b) increased rate of transcription initiation, (c) increased HER-2/*neu* messenger RNA transcript stability, (d) enhancement of translation efficiency from altered HER-2/*neu* mRNA transcript, and (e) decreased turnover of protein product. My studies will address transcriptional and post-transcriptional mechanisms for upregulation of HER-2 in SKOV-3, and, in

particular, the role of alternative RNA processing in upregulating HER-2 protein expression.

Increased mRNA stability can lead to accumulation of message and, thus, increased translational capacity, resulting in protein overexpression. Intrinsically altered HER-2 message stability has not yet been reported, but could result from changes affecting: (1) the length of the 3'untranslated region (3'UTR), (2) sequences within the 3'UTR that confer RNA stabilization, and (3) binding sites for stabilizing proteins. Possible targets capable of having such effects would include: AU-rich sequences in the 3'UTR (10), deadenylation signals, decapping signals (11), affinity binding sites for poly-A-binding protein (PAB) that may stabilize mRNA and also enhance translational efficiency (12), and altered stem-loop structures in 5'UTR and/or 3'UTR that may affect degradation rate and/or translation efficiency (11-13).

Alternative HER-2/*neu* mRNA transcripts have been identified in both human tumors and tumor cell lines (7, 17, 20). Of interest, the SK-OV-3 human ovarian cancer-derived cell line, which produces the highest level of p185^{HER-2/*neu*} and is frequently used as a model cell line in studies of HER-2 overexpression, displays an abundant mRNA species of approximately 8 kb, in addition to the well-characterized and cloned 4.5 kb HER-2/*neu* transcript normally seen. The fact that this alternative transcript is expressed over-abundantly and appears to be selected for in this aggressive cancer cell line suggests that it may be contributing to tumorigenesis. One possibility is that this alternative transcript may facilitate overexpression of HER-2/*neu* via increased translation of p185^{HER-2/*neu*}, thus contributing to tumorigenesis. In order to determine the function of this alternative transcript, it will be necessary to determine its entire sequence and translation product. The study, undertaken, is aimed at structurally and functionally characterizing this alternative 8 kb HER-2/*neu* transcript in SK-OV-3 cells, in terms of (1) identifying sequence alterations that potentially contribute to its accumulation in the cell via increased mRNA stability, and possibly increased translation efficiency, both resulting in p185^{HER-2/*neu*} overexpression; (2) determining its mechanism of generation; and (3) investigating developmental expression patterns.

Preliminary data supports the hypothesis that the alternative 8 kb HER-2/*neu* transcript confers increased p185^{HER-2/*neu*} expression in that: (1) RT-PCR and 5'RACE demonstrate that it contains similar 5'UTR and coding sequences to those of the 4.5 kb transcript, suggesting that it has a similar transcription initiation rate and codes for p185^{HER-2/*neu*}; (2) it displays an elevated half-life in the cell with respect to the 4.5 kb transcript, suggesting that the 8 kb HER-2 is a stable, actively translated transcript and does not contain nonsense mutations, which are known to destabilize mRNA (11); and (3) it is a natural HER-2 gene product due to alternative polyadenylation site usage and not a result of a gene rearrangement.

Purpose of the Present Work

The purpose of my current thesis project, as a DOD predoctoral training fellowship recipient, is to elucidate the role of alternative mRNA transcripts for HER-2/neu with respect to p185^{HER-2/neu} overexpression in human breast and ovarian cancers.

BODY

Experimental Methods, Assumptions, and Procedures

Based on preliminary data (described in my 1996 and 1997 annual reports) and previous reports (17), I have proceeded with the characterization of the aberrant 8 kb HER-2 transcript under the assumption that it is translated into functional p185^{HER-2/neu}. In addition, I have collected evidence that further supports this assumption.

Cell culture. Human breast and ovarian carcinoma cell lines were obtained from American Type Culture Collection (Rockville, MD) maintained in either DMEM (SKBR3 and SKOV-3) or RPMI (BT474), supplemented with 10% fetal bovine serum and 0.05% gentamycin, as described (Lin and Clinton, 1991). All media and supplements were obtained from GIBCO BRL (Life Technologies, Gaithersburg, MD). Unless specified, all other chemicals were purchased from Sigma.

RNA extraction. Cells grown to 80% confluence on 15 cm plates were extracted with TriReagent (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer's protocol, to obtain RNA. RNA was resuspended in RNA sample buffer (50% formamide, 1.5% formalin, 0.1% SDS, 1mM EDTA, 10mM Tris pH 7.4) for mRNA fractionation and Northern blotting, or in 10mM Tris-EDTA, pH 8.0, for reverse transcription and cDNA library construction. RNA concentrations were determined spectrophotometrically at OD₂₆₀.

Isolation of the 8 kb HER-2/neu transcript for analysis of sequence content. Purification of SK-OV-3 mRNA and subsequent size fractionation is required in order to study the 8 kb HER-2 transcript independently of the normal-sized 4.5 kb transcript. To isolate the 8 kb from the 4.5 kb HER-2 transcripts, I modified the method of electroelution from agarose gels described by O'Callaghan et al. (1991, ref. 19; method as described in 1997 DOD report and schematicized in Appendix B). I electroeluted mRNA transcripts by size from denaturing (formaldehyde-containing) 0.7 % agarose-MOPS gels and subsequently dialyzed away the formaldehyde for use of transcripts as templates for downstream enzymatic reactions.

Polymerase chain reaction . Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used to directly obtain alternative sequence information from the 8 kb HER-2 transcript. Fractionated mRNA was reverse-transcribed into first strand cDNA using GIBCO BRL (Life Technologies) Superscript RTII System. First strand cDNAs were used as template for amplification via the polymerase chain reaction using HER-2 sequence-specific primers. Templates were amplified in a Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer Cetus, Emeryville, CA) using the Expand High Fidelity PCR System (Boehringer Mannheim) with 1X High Fidelity PCR buffer containing 2.5 mM MgCl₂, 5 mM of each primer, and 200 mM dNTPs. All primers were obtained from GIBCO BRL (Life Technologies). Numbering of nucleotide and amino acid residues is according to the HER-2 cDNA sequence reported by Coussens et al. 1985 (1). Cycling parameters were: 94° C, 30"; 58° C, 45"; 68° C, 3', for 30 cycles.

The region spanning the polyadenylation signal sequence was amplified from genomic DNA of SKOV-3, BT474, SKBR3, and normal human genomic DNA (a gift from Dr. Mike Litt, Oregon Health Sciences University), using a forward primer identical to nucleotides 4301-4323 of HER-2 cDNA and a reverse primer complimentary to unique sequence located approximately 100 bp downstream from the putative polyadenylation signal and not reported in the GenBank sequence for HER-2 cDNA. PCR was performed on DNA prepared according to the procedure of Strauss (1998) with cycling parameters: 94° C, 30"; 62° C, 30"; 72° C, 60", for 25 cycles.

SKOV-3 fractionated cDNA library construction. Fractionated 8 kb mRNA (as above) was used as a template for cDNA library construction using a λZIPLOX cDNA library construction kit with EcoR1 adaptors (GIBCO BRL, Life Technologies). Electrophoresis of first, reverse-transcribed, single-stranded, and second, double-stranded, strand cDNA was performed to assess quality and size of synthesized products. Products were then ligated with EcoR1 adaptors and phage arms, sequentially, at 16° C for 24 h, per manufacturer's protocol. Phage were packaged at room temperature (RT) for 1 h using a Stratagene Gold Plus lamda phage packaging kit. Complete phage were then used at varying dilutions (1:100, 1:10,000, and 1:1,000,000) to infect Y1090 competent cells (provided, GIBCO BRL), and cells were plated on 15 cm 2XLB plates with 2XYT top agar and incubated at 37° C overnight (o/n) for titer. A titer of 6 X 10⁴ pfu per µl was determined and the entire library was plated at ~25,000 pfu per 15 cm plate for screening. 15 cm GeneScreen Plus Hybridization filters (NEN Life Sciences) were use for screening chilled plates (i.e., 4° C X 1 h after o/n incubation at 37° C) via duplicate plaque lifts of 2 min and 8 min incubation at RT. Nucleic acids were fixed to membranes by UV crosslinking in a UV-Stratalinker (Stratagene, Inc., La Jolla, CA) for 30 seconds. Filters were then pre-hybridized for 2 h at 42° C in 30% formamide, 5M NaCl, and 1% SDS with 10 µg/ml herring sperm (hs) DNA as blocking agent. A probe consisting of 10⁸ cpm γ-(32P)ATP end-labelled oligonucleotide, which is identical to nucleotides 4258-4276 of HER-2 cDNA (1), was added and filters were hybridized o/n (~16-24 h) with hs DNA carrier. Filters were washed in several changes of 0.2XSSC at 55° C, wrapped in polyvinyl chloride and

exposed to Kodak X-AR film o/n at -80°C with intensifying screens. Film was developed in a Kodak M35A X-OMAT Processor. Positive clones were isolated by picking plaques corresponding to duplicate signals on filters and placed in 0.5 ml SAM buffer for 8 h at 4°C . A single μl of this material was then used to reinfect Y1090 cells and the process repeated to obtain a pure population of the isolated positive clone of interest. DNA was then isolated from pure clones by infecting D12S cells (provided, GICO BRL), plating on 10 cm LB plates with 30 $\mu\text{g/ml}$ kanamycin, 0.1 $\mu\text{g/ml}$ IPTG and 35 μl of 5% X-gal and subsequently growing a single white colony o/n in the presence of kanamycin at 37°C . λZL plasmid DNA was purified via Qiagen miniprep kit and sequenced using m13 forward and reverse primers.

Northern blotting was performed using SK-OV-3 total RNA, extracted via Triazole, and subsequently enriched for poly-A⁺ RNA via oligo-dT column chromatography (Qiagen). Nucleic acids were electrophoresed on a 0.8% agarose-formalin MOPS gel and transferred by capillary action onto Gene Screen Plus Hybridization Transfer Membrane (NEN Research Products, Boston, MA) in 10XSSC. Nucleic acids were fixed to membranes by UV crosslinking in a UV-Stratalinker (Stratagene, Inc., La Jolla, CA), and the membranes were blocked in hybridization buffer (50% formamide, 5X SSC, 1% SDS, 10 mg/ml herring sperm DNA) at 42°C for 2 h. Hybridization was 10^7 cpm of random primed $\alpha(32\text{P})\text{dCTP}$ -labelled probe of either a 570 bp HER-2 cDNA 5' coding region-specific fragment (Figure 2, left panel) or a 280 bp HincII cDNA fragment of the unique sequence obtained from an SKOV-3 cDNA library clone (Figure 2, right panel), synthesized using a Random Prime DNA Labelling Kit (Boehringer Mannheim). Probe was added directly to hybridization buffer and agitated incubation was at 42°C for 16 h. Blots were washed in several changes of 0.2XSSC with 0.1% SDS at 55°C for at least 1h. Wet blots were wrapped in polyvinylchloride and analyzed by phosphorimaging (Molecular Dynamics).

A prepared Northern blot of human fetal tissues (Clontech) was hybridized with an RNA probe generated by blunt-ligation cloning of the PCR amplification product obtained from amplification spanning the known polyadenylation signal of the HER-2 3'UTR (PCR described below) into pCR-SCRIPT (Stratagene; La Jolla, CA). The plasmid was sequenced by the Vollum Institute Core Sequencing Facility (Portland, OR) with m13 forward and reverse primers to verify the sequence of the insert and the fidelity of the PCR. An antisense RNA probe complimentary to the region spanning the known polyadenylation signal was transcribed from 1 mg of HindIII-linearized template using ($\alpha\text{-}^{32}\text{P}$)CTP, T7 RNA polymerase, and the T7/SP6 Riboprobe Synthesis System (Promega, Madison, WI). This probe was added to pre-hybridization buffer, as above, and incubation was with shaking at 65°C for 16 h. The blot was washed at 65°C in several changes of 0.1XSSC with 0.1% SDS for 2h.

Results and Discussion

The 5'UTR of the 8 kb HER-2 transcript is similar to 4.5 kb HER-2 5'UTR

Because 5'UTR sequence structure may confer altered mRNA stability and/or translatability (12, 13) and may reflect a gene rearrangement or translocation event (13, 15), I investigated sequences in the 5'UTR of the 4.5 and 8 kb HER-2 transcripts using RT-PCR and 5'Rapid Amplification of cDNA Ends (5'RACE). First stranded (single stranded) cDNAs synthesized from 8 kb fractionated mRNA (as described previously in my 1997 annual report) were tailed at the newly-generated 3' end (i.e. that being complementary to the extreme 5'UTR) with poly-deoxyadenine using terminal deoxytransferase (Boehringer Mannheim). An oligo-dT was used as a forward primer (i.e., to amplify from the newly-synthesized poly-dA tail) and a HER-2 sequence-specific reverse primer identical to nucleotides 188-207 of HER-2 cDNA (1) was used in the PCR.

Figure 1 (left panel) shows a representative ethidium bromide-stained, UV-illuminated 2% agarose gel on which HER-2 5'RACE amplification products of: reverse transcribed SK-OV-3 mRNA from the 4.5 kb fraction (lane 1), the 8kb fraction (lane 2); and no DNA (control, lane 3) were electrophoresed, revealing a band of ~215 bp for amplification products in lanes 1 and 2, which is the size expected for the 5'UTR targeted amplification of the 4.5 kb 'wild-type' transcript. The right panel of figure 1 is a Southern blot of this gel, hybridized with a HER-2 sequence-specific oligonucleotide probe identical to nucleotides 145-163 of the HER-2 cDNA (1) within the region to be amplified (i.e., just 5' to the inner PCR primer). These results were interpreted to indicate that the 5'UTR of the 8 kb transcript is similar to that of the 4.5 kb 'wild-type' transcript, suggesting that no translocation event has occurred affecting 5'UTR sequence. Although a translocation affecting the promoter region cannot be ruled out, it is less likely given a similar 5'UTR. This result would, therefore, indicate that transcription initiation rate, as well as translation initiation rate, is unchanged for the 8 kb transcript, assuming that no point mutations are present within the 5'UTR that would affect either of these rates.

The 3'UTR of the 8 kb HER-2/neu transcript is extended and contains unique sequence

Because 3'RACE consistently gave no amplification product from an 8 kb fraction of SKOV-3 mRNA (see 1997 DOD annual report), in order to determine the unique sequence of the aberrant 8 kb HER-2/neu mRNA transcript expressed in SK-OV-3 cells and to clone its cDNA form, an 8 kb-enriched SKOV-3 cDNA library was constructed in λ ZIPLOX (Gibco BRL, Life Technologies), using, as starting material, size-fractionated mRNA (purified as described above and depicted schematically in Appendix B). Since I had previously found the 8 kb transcript to have an increased half life in SKOV-3 and because many studies have implicated a role for the 3'UTR in mRNA stability (10-15), I targeted the 3'UTR in screening my SKOV-3 cDNA library for the 8 kb HER-2 transcript. Also, since other preliminary data indicated that the 5'UTR and coding regions were similar, I decided to aggressively pursue sequence determination in the 3'UTR region. Using an oligonucleotide probe

identical in sequence to the extreme 3'UTR of HER-2 cDNA, seven clones were isolated and sequenced. Six of the seven clones contained partial coding and 3'UTR sequence of HER-2 cDNA consistent with the reported sequence for the 4.5 kb transcript (1), yet terminating before the polyadenylation signal sequence. A single clone of approximately 7 kb was isolated and found to contain HER-2 3' coding sequence and 3'UTR identical to that of HER-2 cDNA from nucleotides (nt) 3138 to 4531, including the termination codon at nt 3916-3918. The sequence of this clone diverges 13 nt following the putative polyadenylation signal, which is reported to be utilized in generation of the 4.5 kb transcript. Partial sequence of this clone is shown in Figure 2, indicating the polyadenylation signal (underlined) which is identical to that reported for the 4.5 kb transcript (1). The sequence continues for another 2 kb following the reported site of polyadenylation for the 4.5 kb transcript. The extended sequence is unique in that no homologs were identified in a GenBank database search. Furthermore, analysis of the sequence using Blast and DNASTAR computer software programs show that it contains multiple termination codons in all six reading frames, indicating that it is untranslated.

To verify that this sequence was from the 8 kb transcript for HER-2 and not a genomic DNA contaminant or artifact of cloning, a Northern blot of SKOV-3 and T47D mRNA was probed with the unique sequence (Fig. 3, right panel) and with 5'HER-2 coding sequence, as a control (Fig. 3, left panel). A 280 bp HincII fragment of the unique sequence showed hybridization only with the 8 kb transcript in SKOV-3. No hybridization with the unique sequence was seen in mRNA from T47D, a carcinoma cell line that does not express an alternative HER-2 transcript, indicating that the sequence is not a generic artifact of cloning an 8 kb fraction of mRNA, rather that it, in fact, represents sequence of the alternative 8 kb HER-2 transcript expressed in SKOV-3.

The extended 3'UTR of the 8 kb HER-2/neu transcript results from alternative polyadenylation site usage

To determine whether this alternative HER-2/neu transcript is a product of gene rearrangement or alternative splicing. By PCR, using primers that flank the point of divergence of the aberrant sequence in the 8 kb cDNA, the region spanning the putative polyadenylation signal, from the reported HER-2 3'UTR sequence extending 3' through unique sequence obtained from 8kb cDNA library clone, was amplified from SK-OV-3 genomic DNA and control DNA (normal human genome), as well as genomic DNA from BT474 and SKBR-3, two breast cancer cell lines that overexpress HER-2 and have amplification, yet do not express an aberrant HER-2 transcript nor exhibit gene rearrangement. PCR products were electrophoresed on 2% agarose, ethidium bromide-stained and UV-illuminated (Figure 4). If the aberrant transcript is a result of alternative RNA processing, then I expect the sequence to be contiguous in the genome, due to read-through of the consensus polyadenylation site. I expect the PCR to amplify the same sized fragment from both SK-OV-3 and from genomic DNA extracted from any cell line or tissue (given that it does not have a mutation or rearrangement at this site). Conversely, if this aberrant transcript results from a

gene rearrangement, then I expect to amplify the divergent sequence directly from only SK-OV-3 genomic DNA. If contiguous, the expected size for the fragment amplified is 312 bp, which is the approximate size of the product obtained from all genomic DNA templates (Figure 4). As anticipated, no product was obtained without DNA template (see "no DNA" lane, Fig. 4). This result indicates that the 8 kb HER-2 transcript results from an extension of the HER-2 transcript through the first, and likely also the second (see underlined poly-A sites in Figure 2), consensus polyadenylation signal(s), and is a direct product of the "wild-type" HER-2/*neu* proto-oncogene.

An 8 kb HER-2 mRNA transcript is not expressed at detectable levels in human fetal tissues

Because my results have shown the 8 kb transcript to be a natural HER-2 gene product, it displays increased mRNA stability in SKOV-3, and because the putative polyadenylation site reported for the 4.5 kb transcript (1) slightly differs from the reported consensus sequence of 5'-AAATAAA-3' required for polyadenylation signalling (12), it might have a role in enhanced developmental expression of p185HER-2/*neu*. Therefore, to assess human developmental patterns of HER-2/*neu* mRNA expression, a prepared Northern blot of selected fetal tissues was probed with radiolabeled RNA complimentary to the HER-2 3'UTR sequence spanning the reported putative polyadenylation signal and containing sequence complimentary to unique sequence obtained for the 8 kb 3'UTR. No hybridization with an 8 kb HER-2 mRNA was detected despite abundant signal for the 4.5 kb transcript (Figure 5). These preliminary results indicate that the 8 kb HER-2 transcript may not be expressed at significant levels in developing human tissues. However, the possibility exists that this transcript for HER-2 may be exclusively during certain stages of development and in selected human tissues which may not be represented in this particular Northern blot.

CONCLUSIONS

Implications of Completed Research

My preliminary results (including data reported in 1996 and 1997 annual reports) show that (1) the 8 kb HER-2 transcript exhibits increased mRNA stability in SK-OV-3 cells, (2) the ratios between mRNA and protein overexpression in SK-OV-3 cells suggest that this 8 kb transcript is translated into p185HER-2/*neu*, (3) the 8kb HER-2 transcript contains similar sequences in the 5'UTR and coding region to that of the 4.5 kb transcript, which encodes p185HER-2/*neu*, (4) the 3'UTR of this transcript is different from that of the 4.5 kb, and (5) the 8 kb transcript results from alternative polyadenylation site usage and is not a product of gene rearrangement in SKOV-3.

Future Work

Specific Aim #1. I will obtain the remainder of alternative sequence information contained within the larger, aberrant 8 kb HER-2 transcript. Because I have not yet obtained the full downstream sequence including the polyadenylation signal and poly-adenine tail of the 8 kb transcript, I will continue to perform amplification via the polymerase chain reaction (3'RACE), with the goal of subsequent subcloning and sequencing. Reverse transcription (to form cDNA) coupled with polymerase chain reaction (RT-PCR) using forward primers targeting specific amplification of the 8 kb HER-2 3'UTR and oligo-dT as a reverse primer will be performed. Relevant 3'RACE PCR products of the 8 kb HER-2/*neu* transcript cDNA sequence (i.e., those that hybridize with HER-2 probes upon Southern blotting, indicating that they contain relevant HER-2 sequence) will be restriction digested, rerun on a DNA-preparatory agarose gel, excised according to size, and eluted for ligation into pSK (Bluescript) vectors, and directly sequenced using pSK-specific primers.

Novel sequence obtained through the RT-PCR and subcloning experiments described will be entered in the database GenBank and compared for sequence homology with any currently known segments of DNA or RNA that may elucidate the functional significance and origin of the alternative transcript sequence.

Potential difficulties in this methodology that may lead to artifactual PCR products and/or misleading results would be if the remainder of the larger HER-2 transcript has complex secondary structure that inhibits reverse transcription, may generate artifactual PCR products and possibly even inhibit polymerase elongation, making it difficult to amplify.

If I cannot obtain the aberrant sequence via RT-PCR and 3'RACE, I will revert to further screening of the λ ZIPLOX partial SKOV-3 cDNA library. To do this, I will reverse transcribe only the 8 kb size-fractionated, purified mRNA in order to form cDNA. I will ligate this cDNA with adapters and clone into λ gt10. I will then directly sequence all clones that hybridize with a full-length HER-2 cDNA probe. Next, I will generate full-length cDNA clones of the aberrant 8 kb HER-2 mRNA transcript by restriction mapping of partial cDNA cloned sequences and ligate this full-length cDNA into a Bluescript pSK (Stratagene) vector for further analysis.

Specific Aim #2. I will determine the translation product of the 8 kb HER-2 transcript. In order to assess whether the 8 kb mRNA is translated and to characterize the protein product of this alternative transcript, I will subclone the full-length 8 kb cDNA into an appropriate expression vector for in vitro transcription-translation experiments, using the p9002 HER-2 expression vector as a control. I will first standardize the amount of protein translated to the level of transcript synthesized in vitro by incorporating α (³²P)CTP into in vitro transcription reaction and electrophoresing products on a denaturing agarose gel, which will be exposed to autoradiography.

Specific Aim #3. I will verify assessment of expression of the 8 kb HER-2 transcript during human development in a variety of fetal and adult tissues. My preliminary experiments using a prepared Northern blot (Clontech) showed no expression of the 8 kb HER-2 transcript in fetal brain, lung, liver, and kidney. This Northern blotting procedure should be repeated to analyze further fetal, as well as adult, human tissues to assess whether the 8 kb transcript is expressed at all during normal human development. Although analysis of tissues at various stages during development would be useful, it would be virtually impossible to obtain such material; therefore, that route of investigation will not be pursued in this study. Furthermore, if no additional findings of HER-2 8 kb transcript expression in human tissues are encountered, I would not pursue extensive analysis of human breast and ovarian primary tumor tissues, as such pursuits would likely yield no positive results, given that abundant expression of this alternative transcript does not occur in any other tumor cell lines yet investigated.

References

1. Coussens, L., Yang-Feng, T.L., Liao, Y.-C., Chen, E, Gray, A., McGrath, J., Seeburg, P.H., Libermann, T.A., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science* 230: 1132-9, 1985.
2. Ben-Levy, R., Paterson, H.F., Marshall, C.J., and Yarden, Y. A single autophosphorylation site confers oncogenicity to the neu/erbB-2 receptor and enables coupling to the MAP kinase pathway. *The EMBO Journal* 13(14): 3302-11, 1994.
3. Hynes, N. E. and Stern, D. F. The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochimica et Biophysica Acta* 1198: 165-84, 1994.
4. Hung, M.-C., Zhang, X., Yan, D.H., Zhang, H.-Z., He, G.-p., Zhang, T.-q., and Shi, D.-r. Aberrant expression of the c-erbB-2/neu protooncogene in ovarian cancer. *Cancer Letters* 61: 95-103, 1992.
5. Kanai, Y., Ochiai, A., Shibata, T., Oyama, T., Ushijima, S., Akimoto, S., and Hirohashi, S. *C-erbB-2* gene product directly associates with β -catenin and plakoglobin. *Biochemical and Biophysical Research Communications* 208(3): 1067-72, 1995. Ochiai, A., Akimoto, S., Kanai, Y., Shibata, T., Oyama, T., and Hirohashi, S. *c-erbB-2* gene product associates with catenins in human cancer cells. *Biochemical and Biophysical Research Communications* 205(1): 73-8, 1994.
6. Shibata, T., Ochiai, A., Kanai, Y., Akimoto, S., Gotoh, M., Yasui, N., Machinami, R., and Hirohashi, S. Dominant negative inhibition of the association between β -catenin and c-erbB-2 by N-terminally deleted β -catenin suppresses the invasion and metastasis of cancer cells. *Oncogene* 13: 883-9, 1996.
5. Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A., and Press, M.F. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 24: 707-12, 1989.
6. Muller, W.J., Sinn, E., Pattengale, P.K., Wallace, R., and Leder, P. Single step induction of mammary adenocarcinoma in transgenic mice bearing activated *c-neu* oncogene. *Cell* 54: 105-115, 1988.
7. Tyson, F.L., Boyer, C.M., Kaufman, R., O'Briant, K., Cram, G., Crews, J.R., Soper, J.T., Daly, L., Fowler, W.C., Jr., Haskill, J.S., and Bast, R.C., Jr. Expression and amplification of the HER-2/neu (*c-erbB-2*) protooncogene in epithelial ovarian tumors and cell lines. *American Journal of Obstetrics & Gynecology* 165(3): 640-6.
8. Lonn, U., Lonn, S., Nilsson, B., and Stenkvist, B. Intratumoral heterogeneity for amplified genes in human breast carcinoma. *Internal Journal of Cancer* 58: 40-5, 1994.
9. Vu, T.H., Martin, G., Lee, P., Mark, D., Wang, A., and Williams, L.T. Developmentally regulated use of alternative promoters creates a novel platelet-derived growth factor receptor transcript in mouse teratocarcinoma and embryonic stem cells. *Molecular and Cellular Biology* 9(10): 4563-7, 1989.
10. DeMaria, C.T., & Brewer, G. AUF1 binding affinity to A+U-rich elements correlates with rapid mRNA degradation. *The Journal of Biological Chemistry* 271(21): 12179-84, 1996.
11. Beelman, C.A. & Parker, R. Degradation of mRNA in eukaryotes. *Cell* 81: 179-83, 1995.
12. Jackson, R.L. & Standart, N. Do the poly(A) tail and 3'untranslated region control mRNA translation? *Cell* 62: 15-24, 1990.

13. Sonnenburg, N. mRNA translation: Influence of the 5' and 3' untranslated regions. *Current Opinions in Genetics and Development* 4: 310-15, 1994.
14. Tanguay, R.L. and Gallie, D.R. Translational efficiency is regulated by the length of the 3' untranslated region. *Molecular and Cellular Biology* 16(1): 146-56, 1996.
15. Sachs, A.B. Messenger RNA degradation in eukaryotes. *Cell* 74: 413-21, 1993.
16. Collins, V.P. Epidermal growth factor gene and its transcripts in glioblastomas. Recent Results in *Cancer Research* 135: 17-24, 1994. Callaghan, T., Antczak, M., Flickinger, T., Raines, M., Myers, M., and Kung, H.-J. A complete description of the EGF-receptor exon structure: implication in oncogenic activation and domain evolution. *Oncogene* 8: 2939-48, 1993. Wong, A.J., Ruppert, J.M., Bigner, S.H., Grzeschik, C.H., Humphrey, P.A., Bigner, D.S., and Vogelstein, B. Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proceedings of the National Academy of Sciences, USA* 89: 2965-69, 1992. Ekstrand, A.J., Sugawa, N., James, C.D., and Collins, V.P. Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. *Proceedings of the National Academy of Sciences, USA* 89: 4309-13, 1992. Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Libermann, T.A., Schlessinger, J., Downward, J., Mayes, E.L.V., Whittle, N., Waterfield, M.D., and Seeburg, P.H. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309: 418-25, 1984.
17. Fujimoto, J., Hori, M., Ichigo, S., Misao, R., and Tamaya, T. Preliminary study of oncogene expressions in endometrial cancers: Aberrant estrogen receptor gene and erbB-2 expressions. *European Journal of Gynaecological Oncology* 16(1): 40-7, 1995. Karlan, B.Y., Jones, J., Slamon, D.J., and Lagasse, L.D. Glucocorticoids stabilize HER-2/neu messenger RNA in human epithelial ovarian carcinoma cells. *Gynecologic Oncology* 53: 70-77, 1994. Jones, J., Lagasse, L.D., and Karlan, B.Y. Steroid hormone independence of HER-2/neu mRNA expression in four human ovarian cancer cell lines. *Gynecologic Oncology* 55: 421-6, 1994. Scott, G.K., Robles, R., Park, J.W., Montgomery, P.A., Daniel, J., Holmes, W.E., Lee, J., Keller, G.A., Li, W.-L., Fendly, B.M., Wood, W.I., Shepard, M., and Benz, C.C. A truncated intracellular HER2/neu receptor produced by alternative RNA processing affects growth of human carcinoma cells. *Molecular and Cellular Biology* 13(4): 2247-57, 1993. Hung, M.-C., Zhang, X., Yan, D.-H., Zhang, H.-Z., He, G.-p., Zhang, T.-q., and Shi, D.-r. Aberrant expression of the c-erbB-2/neu protooncogene in ovarian cancer. *Cancer Letters* 61: 95-103, 1992. Lichtenstein, A., Berenson, J., Gera, J.F., Waldburger, K., Martinez-Maza, O., and Berek, J.S. Resistance of human ovarian cancer cells to tumor necrosis factor and lymphokine-activated killer cells: Correlation with expression of HER-2/neu oncogenes. *Cancer Research* 50: 7364-70, 1990. Zhang, X., Silva, E., Gershenson, D., and Hung, M. Amplification and rearrangement of c-erbB proto-oncogenes in cancer of human genital tract. *Oncogene* 4: 985-9, 1989.
18. Karlan, B.Y., Jones, J., Slamon, D.J., and Lagasse, L.D. Glucocorticoids stabilize HER-2/neu messenger RNA in human epithelial ovarian carcinoma cells. *Gynecologic Oncology* 53: 70-7, 1994.
19. Harty, R.N. & O'Callaghan, D.J. Isolation and purification of specific, overlapping mRNA species by electroelution from agarose gels. *Biotechniques* 10(6): 728-9, 1991.
20. Doherty, J.K. and Clinton, G.M. A novel HER-2/neu gene product results from intron retention. Manuscript in Preparation, 1998.

APPENDIX A

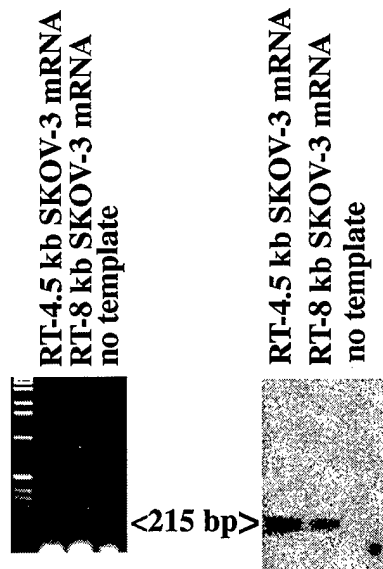


Figure 1. The 5'UTR of the HER-2 8 kb and 4.5 kb mRNA is similar. Reverse-transcribed mRNA fractions from 8 kb and 4.5 kb SKOV-3 mRNA were poly-deoxyadenine tailed using terminal deoxy transferase (Boehringer Mannheim), and subjected to 5'RACE using oligo-dT as a forward primer and a reverse primer complimentary to nucleotides 188-208 of HER-2 cDNA. PCR amplification products were visualized by ethidium bromide staining and UV-illumination following electrophoresis on 2% agarose (left panel). Nucleotides were then transferred to GeneScreen membrane (NEN Life Sciences), hybridized with γ -³²P end-labelled oligonucleotide identical to nucleotides 144-165 of HER-2 cDNA (Coussens et al., 1985), washed and analyzed by phosphorimaging (Molecular Dynamics).

nt 4351 of HER-2 3'UTR (Coussens et al., ref. 1)

↓
.... ATGGTGTCAGTATCCAGGCTTTGTACAGAGTGCTTTTCTGTTTAGTTT
TTACTTTTTTTGTTTTGTTTTTTTAAAGACGAAATAAAGACCCAGGGG
AGAATGGGTGTGTATGGGGAGGCAAGTGTGGGGGGTCTTCTCCAC
ACCACTTTGTCCATTTGCAAAATATATTTTGGAAAACAGCTAAGCACC
GGCCTATGTCTGGGGGTGGATCTTCACAAAGCTTGGATGAAGAACCA
GACATCCAGTTCTCCAGCTTCAGGGTTGGGGGAGATCGGGAACTGGA
TTCACATCTGAGAACCCTTTGACGACCACATCGTTCTTATTTATAAC
GAACTAATTCCTAAAGCCACTCCCAGAAATGGGGTTATTCCTCCTT
TTAATTCAAGAAAAT.....~2 kb..... GGAGAACACCAGCTG
TCCGGTTGGAGAGGTTGACTTTTCTAAGGGGTGGCAGAAGCAGAAGG
AAATCCTTACATATGTAGACTTGCACAGTTCAAACATGTATTGTTCA
AGGATCGCCTGGGTTTATAATATATATTATTTGTATATTTAAATATAC
ATGTTTGTGATTTTTTAAAGGTTTATTTCTGGAACCTTAAAGCAAGAAT
AGAAAACCTTAAATGTGCTAAAAGATTAATAAGCTTTGAGAATTTGTA
GAGTTGATAGCTACACTGGTCACTGATTTAGGAAGGTCCATACCCAT
TTGAATGTCGACGCGGCCGC...(~1.5 kb)..AAATAAA....AAAAAAA-3'

Figure 2. Unique sequence extension 3' to known HER-2 cDNA 3'UTR. Primary sequence is shown at 5' and 3' ends of insert within λ ZIPLOX clone isolated from a SKOV-3 8 kb-fraction-cDNA library. Sequence of the putative polyadenylation signal reported to be utilized for the 4.5 kb transcript is underlined; in addition, a second consensus sequence (also underlined), matching that above, was found approximately 2.5 kb 3' to the first, although it does not appear to be followed by a polyadenine tail. The first divergent nucleotide of the unique sequence is denoted by a line over the letter and is located at the start of the polyadenine tail in the reported sequence for the 4.5 kb transcript.

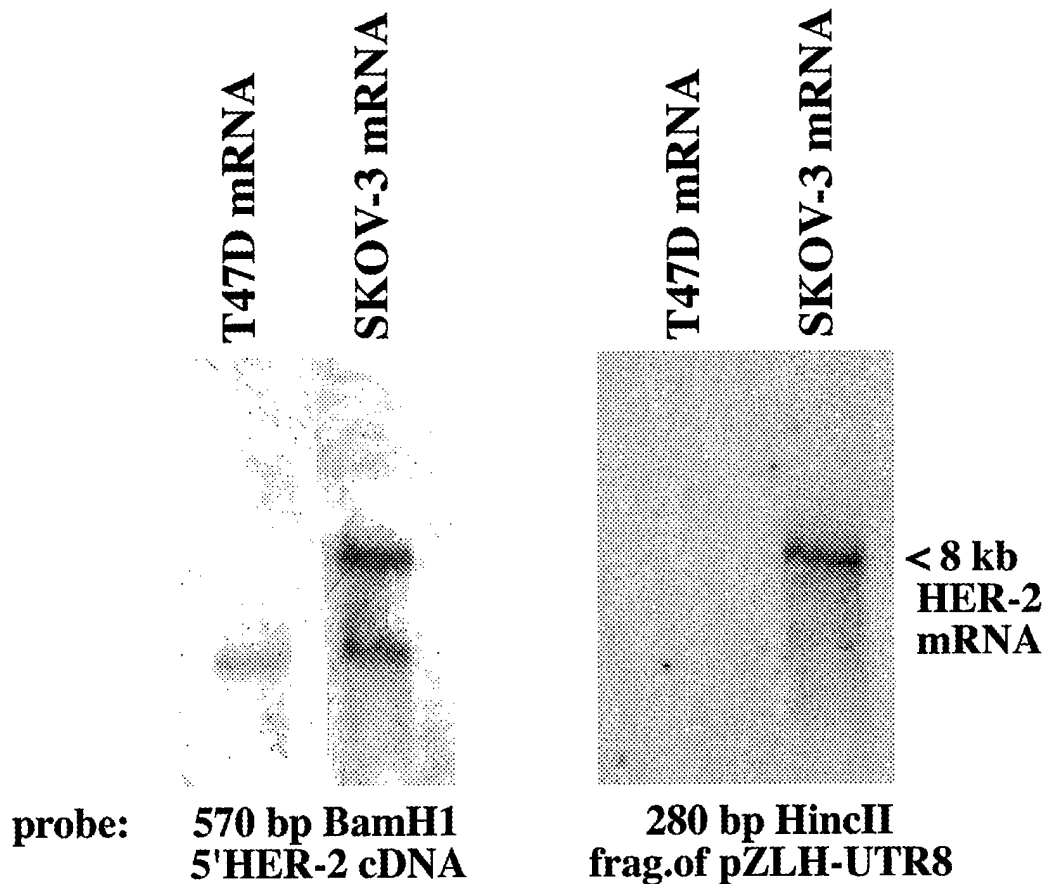


Figure 3. Divergent sequence of an alternative 8 kb HER-2 mRNA is within the 3'UTR. Northern blot of 2 μ g T47D (lanes 1, 3) and SKOV-3 (lanes 2, 4) mRNA (as described) hybridized with a 5'HER-2 cDNA probe, showing HER-2 mRNA of 4.5 kb in both T47D and SKOV-3 (lanes 1 and 2; right panel) and 8 kb message only in SKOV-3 (lane 2). The left panel shows the same Northern blot, after stripping (0.1Xssc at 100 $^{\circ}$ C for 10 min) and re-hybridizing with unique sequence obtained from cDNA library clone of HER-2 message (described in text).

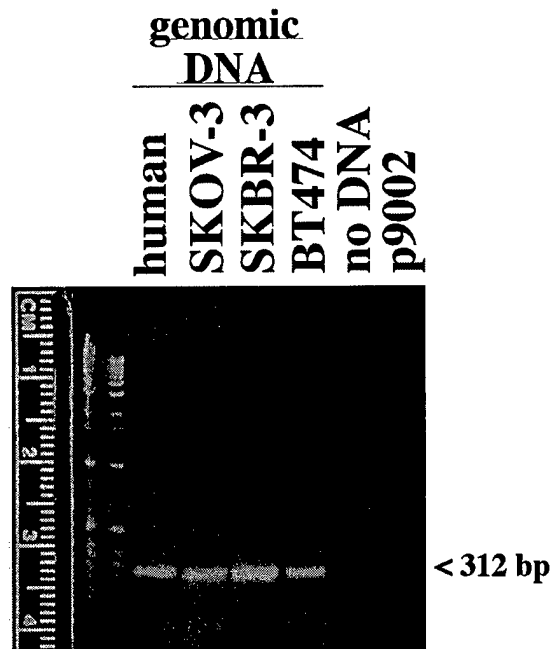
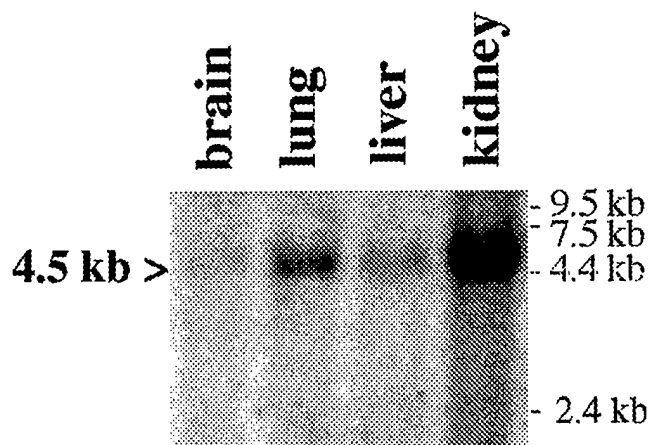


Figure 4. 8kb HER-2 transcript results from alternative polyadenylation signal usage: sequence of 8 kb HER-2 3'UTR is contiguous with terminal HER-2 exon sequence in genomic DNA. Ethidium bromide stained, UV-illuminated gel of PCR products obtained using a forward primer identical to known HER-2 cDNA 3'UTR sequence at nucleotides 4301-4323 and a reverse primer complimentary to sequence obtained from a cDNA library clone of the 8 kb HER-2 transcript located approximately 100 bp 3' of the reported polyadenylation signal.



probe: **HER-2 3'UTR**

Figure 5. HER-2 8 kb mRNA transcript is not developmentally expressed at detectable levels in human fetal tissues which express the 4.5 kb HER-2 transcript. Northern blot of 2 μ g mRNA from human fetal tissues, as indicated (Clontech), hybridized with random prime 32 P-labelled cDNA of HER-2 3'UTR sequence, showing no hybridization with an mRNA of 8 kb.

APPENDIX B

mRNA Fractionation Procedure

