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Amplification of the *HER2* gene occurs in more than 20% of breast cancers and is associated with aggressive tumor cell growth. In addition to gene amplification, genetic mechanisms including translational deregulation may contribute to the overexpression of the HER2 oncoprotein. The HER2 mRNA contains a highly conserved short upstream open reading frame that represses downstream translation. Recent studies have shown that this upstream open reading frame represses translation of HER2 as well as a reporter gene cistron, and that it functions in mammalian cells, in yeast and in cell free translation extracts. Although the analysis of the mechanism by which the upstream open reading frame acts is not yet complete, the very short intercistronic spacing between the upstream open reading frame and the HER2 coding region is a critical component. Ribosomes that have translated the upstream open reading frame are unable to reinitiate efficiently at the HER2 initiation codon. The consequences of this translation mechanism for transforming activity of the HER2 protein synthesis are under investigation.

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Robert Schuler 12/23/97

PI - Signature Date

4. Table of Contents

5. Introduction:

Overexpression of the HER2 oncoprotein occurs in 20-30% of breast tumor cells and is predictive of aggressive tumor growth [1-3] . As well, *HER2* functions as an oncogene in cell culture transformation assays [4-6]. Thus, HER2 may be a causal factor in the development and/or progression of breast cancer.

The mechanism by which HER2 is overexpressed in tumor cells is incompletely understood. Several studies have highlighted a correlation between with *HER2* gene amplification and HER2 protein overexpression (reviewed in [3]). These observations suggest that the abnormal *HER2* gene dosage (i.e. the number of DNA copies) in breast cancer cells may account for HER2 protein overexpression. However, in some tumor cells, transcriptional activation of HER2 occurs without detectable gene amplification [7]. Moreover, our work suggests that translational controls also contribute to the overexpression of HER2 in tumor cells (manuscript in preparation).

In most eukaryotic mRNAs, the most 5' proximal AUG codon serves as the site for initiation of protein synthesis [8] . However, a majority of oncogene transcripts including the HER2 mRNA, as well as many mRNAs encoding growth factors and cellular receptors contain AUG codons and associated short open reading frames (uORFs) upstream from the major translational start site [9]. The role of these uORFs is largely uncharacterized though an increasing number of examples illustrate the potential for these elements to influence quite substantially expression of the downstream reading frame (reviewed in [10]).

In the case of the HER2 mRNA, the uORF inhibits translation of a downstream reporter gene in mammalian cell transfection assays (manuscript in preparation). This research project was designed to elucidate the role of this uORF in breast cancer by two parallel experimental approaches. First, studies using *S. cerevisiae* will clarify the translational regulatory mechanism through which this uORF operates. Second, studies of the uORF effects on the expression and transforming potential of the *HER2* gene will help reveal the role of the uORF in tumorogenesis.

6. Body:

Effects of the HER2 uORF on downstream translation in *S. cerevisiae.*

The first set of studies was designed to assess the effects of the HER2 uORF in yeast. The rationale for these experiments was that if the uORF inhibits translation in yeast as it does in mammalian cells, then it may be possible to utilize a yeast genetic approach to identify and characterize *cis*- and *trans*-acting mutations that alleviate the inhibition.

To investigate the effects of the HER2 uORF in yeast we first constructed a series of yeast expression plasmids that contain portions of the HER2 leader upstream of lacZ sequences (Fig. 1). For each plasmid containing the uORF, we constructed a control plasmid in which the AUG codon of the uORF was mutated to AAG, thereby eliminating the uORF. These plasmids were then transformed into the *S. cerevisiae* strain Cry-1 and ß-gal activity was measured in extracts of transformants.

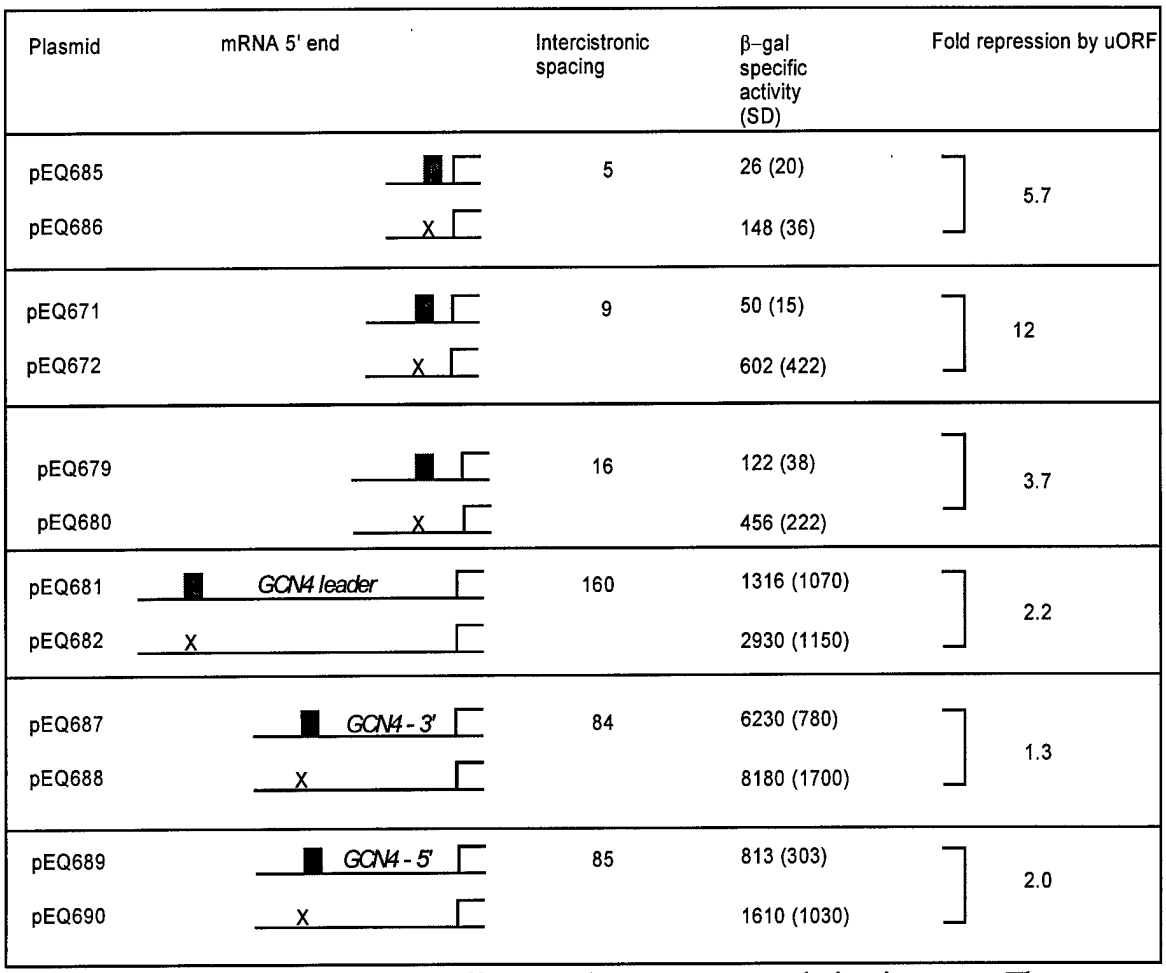

Fig. 1. Analysis of HER2 uORF effects on downstream translation in yeast. The plasmids expressing transcripts having the depicted 5' ends regions were transformed into yeast. ß-gal expression from 5 transformants of each construct was measured. The fold repression is calculated by dividing ß-gal expression from a plasmid containing a mutation in the upstream AUG codon (X) by expression of the corresponding construct containing the uORF (filled rectangle). Oligonucleotide linkers and translationally neutral sequences from the *GCN4* transcript leader [11] were inserted between the uORF and the ß-gal ORF to vary the intercistronic spacing.

In our initial experiments, we found that the HER2 transcript leader repressed ß-gal expression to a very low level (data not shown). However, this inhibitory effect was independent of the uORF since mutation of the uORF AUG codon did not result in increased ß-gal expression. This uORF-independent repressive effect of the HER2 leader was not detected in mammalian cells, a discrepancy we suspect is due to the greater sensitivity of yeast translational machinery to RNA secondary structure.

We next made a series of yeast expression plasmids containing the HER2 uORF and the authentic 5 nucleotide intercistronic region, but none of the HER2 transcript leader upstream from the uORF. As shown in Fig. 1, the uORF repressed ß-gal expression approximately five-fold in this experiment.

We next inserted sequences into the intercistronic region between the HER2 uORF and the ß-gal ORF to test the hypothesis that the short intercistronic spacing between the two uORFs is responsible for the inhibitory effect of the uORF. Lengthening the intercistronic spacing reduced the inhibitory effect of the uORF (Fig. 1). The uORF in constructs with greater than ~80 nucleotides of intercistronic spacing inhibited downstream expression only 2-fold. These data suggest that the inhibitory effect of the uORF in yeast is largely a consequence of ribosomes translating the uORF but being unable to reinitiate at the HER2 AUG codon.

One problem with these experiments was the large variation in absolute levels of ß-gal expression among the pairs of construct (Fig. 1). For example, pEQ688 and pEQ690 are appear quite similar but express 5-fold different levels of ß-gal. The basis for this variation is under investigation. In particular, examination of ß-gal mRNA accumulation in the various transformants should be informative.

Consequences of translational derepression on expression of, and transformation by the authentic HER2 protein.

The second goal of this project is to establish whether the uORF regulates translation of the authentic HER2 protein and to test the prediction that translational derepression of HER2 protein synthesis, resulting from inactivation of the translationally repressive uORF, enhances the transformation efficiency of the HER2 oncoprotein.

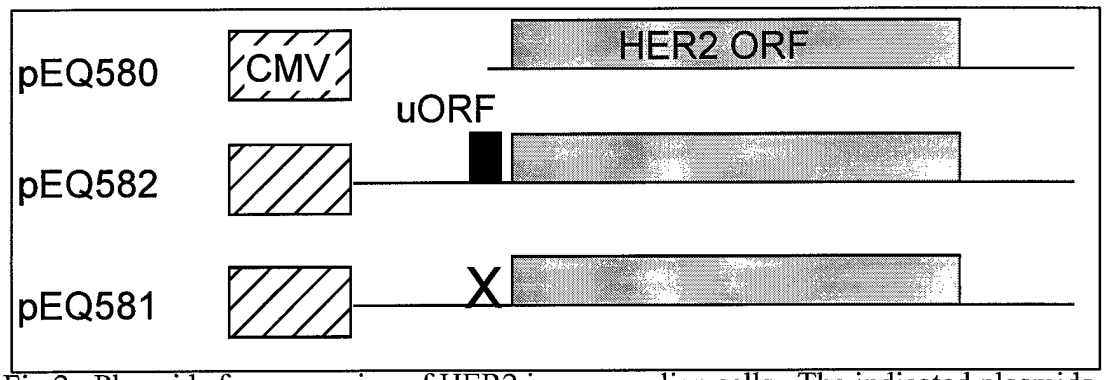

Fig 2. Plasmids for expression of HER2 in mammalian cells. The indicated plasmids were constructed such that the CMV immediate early promoter-enhancer controls expression of mRNAs containing HER with no uORF (pEQ580), the wildtype HER2 transcript leader (pEQ582) or the HER2 transcript leader with a mutation of the uORF AUG codon to AAG (pEQ581).

Previous studies demonstrated that the HER2 uORF repressed downstream translation of ß-gal when *lacZ* sequences replaced the HER2 cistron (manuscript in preparation). To evaluate whether the uORF also repressed HER2 expression in a more authentic mRNA, we constructed plasmids containing the uORF and flanking transcript leader sequences upstream of the HER2 ORF. Two sets of plasmids were constructed in which RNA expression was controlled either by the phage T3 promoter or by the cytomegalovirus major immediate early promoter (Fig. 2), thereby enabling assays of the uORF effect both in cell free assays and *in vivo.*

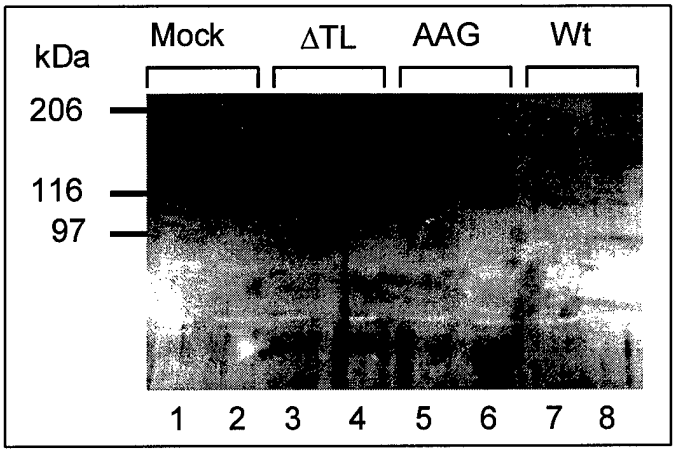

Fig. 3. Immunoblot of HER2 expressed in COS7 cells. COS7 cells were mock transfected, or transfected with HER2 expression plasmids pEQ580 (ΔTL), pEQ582 (AAG) or pEQ581 (Wt). Two (lanes 1,3,5, and 7) and three (lanes 2, 4, 6 and 8) days post transfection, whole cell extracts were prepared by SDS lysis and HER2 expression was analyzed by immunoblot (by Dr. Gail Clinton, OHSU). The migration of protein molecular size markers is indicated on the left. Elimination of the uORF (lanes 3-6) resulted in a substantial increase in the level of HER2 protein above the background level detected in mock transfected cells (lanes 1 and 2) or in cells transfected with the construct containing the wildtype uORF (lanes 7 and 8).

The HER2 uORF repressed translation of HER2 protein in transient transfection assays (Fig. 3). Plasmids were transfected into COS7 cells and whole cell extracts were analyzed by immunoblot analysis. We were unable to detect the HER2 protein using commercially available antiserum. However, Dr. Gail Clinton (Oregon Health Sciences University) analyzed these samples for us using her very sensitive antisera. Results of the immunoblot show that the uORF represses downstream translation in COS7 cells. Thus, the HER2 uORF represses downstream translation of ß-gal or HER2, and both *in vivo* in mammalian cells (Fig. 3) and in cell free translation assays (data not shown).

We next sought to determine whether the reduced expression of HER2 by the uORF resulted in a decrease transforming activity of the HER2 transgene. The simplest hypothesis is that repression of HER2 protein abundance by uORF should reduce transforming activity. However, alternatives need to be considered. For example the HER2 protein expressed in the absence of the uORF may be less active due to misfolding of the nascent polypeptide chain.

To analyze the transforming activity of transfected HER2 constructs, we first attempted to use transformation assays of NIH/3T3 cells, a conventional cell type for this sort of study and a cell type in which HER2-mediated transformation has been demonstrated [4]. Several experiments were conducted in which optimization of (i) carrier DNA preparations, (ii) buffer conditions and (iii) concentrations of serum were used. Nonetheless, we could not identify conditions in which the positive control construct, LTR/erbB2, transformed NIH/3T3 cells above the level seen in mock transfected cells. In discussing these results with others researchers experienced with transformation assays, we learned that these assays may be difficult to reproduce in

NIH/3T3 cells due to a variety of factors including variations among batches of the cells and serum.

We next tested the transformation of rat embryo fibroblasts (Fig. 4). As with the NIH/3T3 cells, we detected a moderate number of foci in the controls and no reproducible increase in the foci number after transfection of HER2.

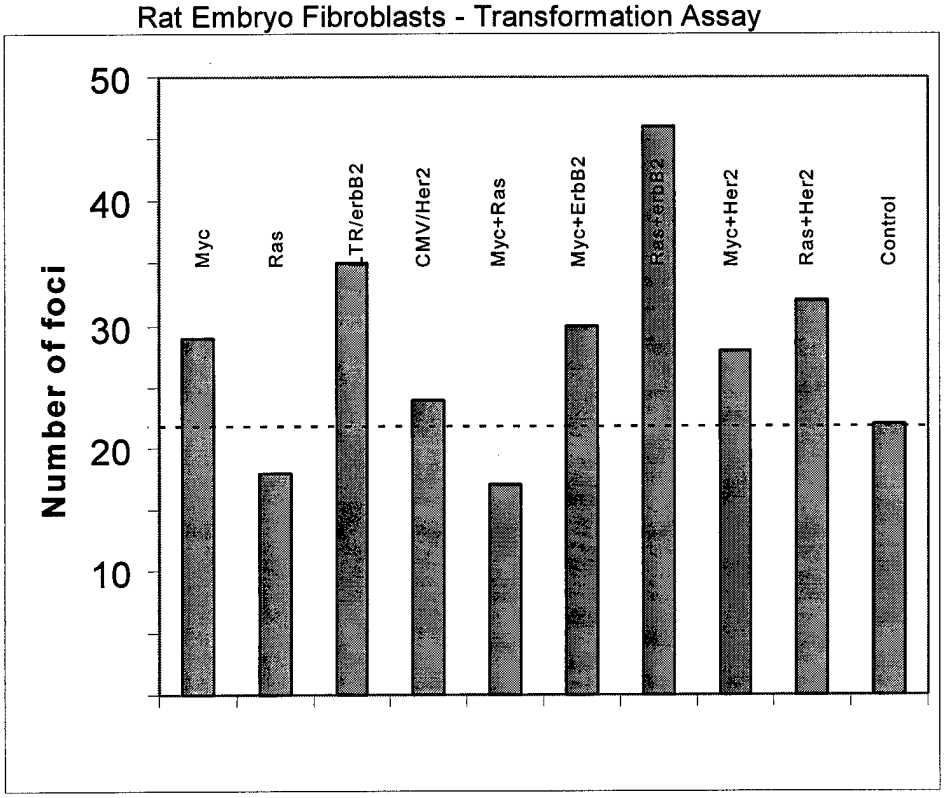

Fig 4. Transformation assay in rat embryo fibroblasts. The indicated plasmids were transfected into rat embryo fibroblasts and foci were counted ~3 weeks later.

Finally, we tested the ability of HER2 to transform 208F cells (Fig. 5). In these experiments, no background foci were detected in cells transfected with negative controls. As well, activated Ras, a positive control, gave a large number of foci. However, neither the HER2 expression plasmid Ltr2/erbB2, that lacks the uORF and was shown to transform NIH3T3 cells [4], nor our CMV-HER2 plasmid (pEQ580) transformed 208F cells. The HER2 expression plasmids did not increase the number of colonies after cotransfection with Ras or Myc. These results suggest the HER2 in not a highly potent transforming oncogene in these cells.

Because of our inability to detect transforming activity of HER2, we have not yet been able to ascertain whether the uORF changes the amount of functional HER2 protein.

Transfected plasmid	Number of Foci
pTLRcMyc	0
Ras (pGEJ)	+++ (~500-1000)
Ltr2/erbB2	0
Her2 (pEQ580)	0
Myc + Ras	+++
Myc + erbB2	0
Ras + erbB2	+++
Myc + HER2	0
Ras + HER2	+++
Control – carrier DNA	0

Fig. Transformation of 208F cells. The indicated plasmids were transfected into 208F cells and foci were counted after staining the monolayers at 3 weeks.

We plan to utilize one or more of several alternative strategies to reveal whether the uORF affect the transforming potential of the *HER2* gene. For example, we will express HER2, with and without the uORF, in MCF-7 cells and determine the effects on growth rate of these cells, similar to the approach employed recently by Pegram *et al.* ([12]).

7. Conclusions:

a) The uORF in the HER2 transcript leader represses HER2 protein expression in mammalian cells, in yeast and in cell free extracts.

b) The short intercistronic spacing between the uORF in the HER2 transcript leader is responsible, at least in part, for the inhibitory effect of the uORF in *S. cerevisiae*.

c) HER2 is not a strong transforming gene. Elucidating the effects of the uORF on transforming efficiency of *HER2* requires further study.

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9. Appendices - none