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TITLE: Antibody Probes to Estrogen Receptor-α Transcript-Specific Upstream Peptides: Alternate ER-α Promoter Use and Breast Cancer Etiology/Outcome

PRINCIPAL INVESTIGATOR: Brian T. Pentecost, Ph.D.

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13. ABSTRACT *(Maximum 200 Words)*

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In the first year we have proof of principal that antibodies can be made against the short upstream peptides but the first generation immunoreagents are neither sensitive enough nor sufficiently specific for either use in evaluating tumors. Studies are proceeding, in line with the original statement of work, to generate stronger immunoreagents. In a complementary area, we have improved our methods for estimation of ER mRNA levels by developing assays which use 'RealTime PCR' in the Roche LightCycler ™. These assays reduce amounts of RNA needed for assays, are faster and are more robust.

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Probes to Estrogen Receptor-α Transcript-specific Upstream Peptides: Alternate ER-α Promoter Use and Breast Cancer Etiology/Outcome. DAMD 17 01 1 0261 Principal Investigator: Pentecost, Brian T.

INTRODUCTION

Positive Estrogen Receptor alpha (ER) status correlates with a reduced incidence of breast cancer recurrence in the first years after resection of tumors, and predicts a favorable response to adjuvant anti-estrogens (1). ER-protein in breast tumors increases with patient age (2). The project develops novel antibody reagents to probe the expression of ER from alternate promoters taking advantage of short peptides encoded in regions of the ER mRNAs that are promoter specific and not shared.

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Task 1. Polyclonal Antisera to Upstream peptides of the alternate Estrogen receptor transcripts

Year 1:

Synthesize peptides.

Initiate generation of Polyclonal antisera in rabbits.

Characterize antisera in Western blots using phased program described in plan.

Evaluate need to modify peptide immunogens based on specificity and sensitivity of preliminary results

Initial immunogens were synthetic peptides representing the translated products of the upstream open reading frames (uORFs) from the alternate ER promoter transcripts (see Fig 1, Fig.1b). The complete distal peptide (13 unique residue sequence, 5 shared with prox-peptide) and a C-terminal truncated prox-peptide containing only the 15 residues not shared with the distal peptide, with the shared WPAGF region not included .

Peptides (5.1 umol) were conjugated at their N-terminal amine with NHS-LC-biotin II (4.1 umol, Pierce) by incubation in PBS, pH 7.4, overnight at room temperature. Solutions were stored refrigerated until use. Prior to injection, an equimolar concentration of streptavidin (based on 4 biotin binding sites per mole) was added and the solution mixed with an equal volume of adjuvant. Each rabbit received approximately 125 nmol s.c. at multiple sites. Injections were normally every four weeks, with samples for ELISA being recovered two week after injection. Animal treatments and numbers were in accordance with our IACUC approved protocol.

The antisera from rabbits #747 and #748 to the distal peptide were able to detect immobilized distal peptide in ELISAs after 2 and more booster immunizations subsequent to the primary immunization (see Figs 2 & 3). The initial data showed cross reaction to a full length proximal peptide: this was a stronger reaction than against the distal peptide and suggested a role for the shared C-terminal regions as the effect was not seen with the shorter prox-peptide lacking shared regions. However, rabbit #747 showed better specificity to the distal peptide after a fourth boost when rabbits were euthanized and serum collected (not shown). We moved to a western blot test as a format which is more informative and which we need to employ in the future. We used the complete distal peptide ORF fused to the C-Terminus in an over-expressed maltose binding protein (MBP, these reagents were developed as alternate immunogens for monoclonal generation, see Task 2 below). This approach was used so that products that were easily resolved by western blot were obtained (the native peptides are only ~2KDa and difficult to analyze by conventional SDS-PAGE and Western blot, the fusion proteins are ca 40KDa).

The serum from rabbit #747 showed good specificity against distal peptide MBP fusion in Western blots (Fig. 4), this antiserum could clearly discriminate between the MBP-distal peptide fusion and MBP protein or a MBP-prox peptide fusion in a western blot. In contrast serum from rabbit #748 showed a non-specific interaction with MBP

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Further analysis showed that the #747 antiserum at a dilution of 1:200 could detect the distal peptide at levels down to at least 25 ng (Fig. 6). This is effectively ~1.25ng of the distal peptide, given that it is ~5% of the fusion protein and we probably need to be able to detect 10X lower levels to detect the peptide in cells based on crude extrapolation of our experience with antisera to the complete ER.

A further test was against a fusion construct of GFP and the distal peptide transfected to mammalian cells (Fig. 7). This construct was described in the original grant application. the N-terminal fusion of the distal peptide ORF to GFP in pEGFP_N1 generates fluorescent protein on cell transfection (not shown) and the fusion is detected in cell extracts in a specific manner by antisera to the GFP moiety (Fig. 7 panel 1). However, the distal peptide antisera #747 fails to specifically detect the distal peptide-GFP fusion in transfected HeLa cells due to heavy cross reaction to cellular proteins (Fig. 7, panel 2, see bands in track of cells not transfected with a distal peptide fusion).

Data with the immune reagents to the distal ORF peptide indicate that antisera can be raised, but our prelimary reagents are not strong enough for use in cell analysis. The antiserum of rabbit #747 will be further purified by immunopurification on columns of the immunizing peptide or the distal MBP fusion and also by depletion of components reactive with Hela proteins, this may give a useable reagents. Work in task 2 builds on our preliminary experience and attempts to develop monoclonal antibodies.

Clearly the data for the distal peptide polyclonal antiserum #747 show that presence of the 5 residues shared by the prox peptide does not automatically lead to cross reaction between prox and distal peptide reagents. The distal peptide is immunogenic but the resulting polyclonal antisera are weak and have difficulty in detecting the distal ORF peptide in transfected cell models. Current work is focused on generating mouse monoclonals and also in trying to isolate a more effective fraction from the rabbit polyclonal #747 using immunoaffintiy chromatography.

Serum from one rabbit (#729) receiving the prox-peptide showed specificity and immunoreactivity against the immunizing peptide after preliminary immunization and two followup immunizations of immunogen by ELISA. The second rabbit receiving the immunogen for the Prox-peptide did not generate a detectable immune reaction specific to the proximal peptide. The immune serum from rabbit #729 after the second and subsequent boosts had the best titre and specificity of all of our initial experimental animals. However, the positive ELISA signal was only with the truncated immunizing peptide; the anti serum *did not* detect a prox-peptide that was complete , ie contained the 5 residues shared with the distal peptide (Fig. 1). Some characteristic of the complete peptide clearly hindered immunoreactivity in an ELISA format.

After four boosts following the initial immunization we euthanized rabbits and collected serum. As an additional test we used the rabbit #729 antiserum in Western immunoblots with the complete prox-peptide ORF fused to the C-Terminus of over-expressed Maltose binding protein. Once again the #729 antiserum failed to detect a complete prox-peptide (Fig. 5). The short prox peptide is clearly immunogenic and some of the data with the distal petide antisera (cross reaction problems) justifies our initial attempt to make antisera against only the unique region. In line with the statement of work we have reviewed our selection of immunogens relevant to the prox peptides and have immunized mice with full length prox peptide and with the MBP-fusion proteins.

Task 2 Monoclonal (mAb) reagents against Upstream peptides of the alternate Estrogen Receptor transcripts

Year 1:

Initiate generation of monoclonals: schedule fusions

Expand clones test supernatants

Select preliminary group of positive clones for analysis based on immunoreactivity in ELISA & BIAcore

We currently have groups of mice under treatment with two distinct immunogens for each of the two peptides. The extent to which the mice have generated an immune response will be evaluated after 5 weeks, ie in June 2002.

From these results we will select a limited group of immunogens/mice to use as sources of splenic tissue for the monoclonal fusions.
The peptides used as immunogens were synthetic peptides to entire uORF regions without conjugation to carriers and, separately, peptides as C-terminal fusions to the E. *coli* Maltose Binding protein (MBP) expressed in E. *coli* and purified on amylose resin (see below).

Synthetic peptides immunogens: The synthetic peptides represent a minimalist approach, the only positive clones should be against the peptides and we will have to determine if mAbs cross react due to shared regions. We are using the Proximal promoter uORF peptide as an intact 20 residue synthetic peptide, this was a modification from the peptide used for rabbit polyclonals. The Peptide is now the complete uORF rather than the shorter unique regions. This selection was made because: 1. We were able to use an intact distal peptide with a C-terminal shared region to raise an immune reagent that did not cross react to the complete proximal peptide nor with an MBP fusion with the complete prox peptide (Fig 4). 2. The polyclonal antiserum against the truncated proximal peptide containing only the unique15 residues interacted only with the immunizing peptide, it failed to work in ELISAs and western immunoblots with the complete peptide.

Fusion Proteins: Peptides were expressed as C-terminal fusion proteins in E. *coli* using fragments inserted to pMAL-c2. pMalC2 is an expression vector where the malE gene encoding a Maltose binding protein (MBP) is under the control of pTac; an IPTG inducible promoter. The uORF sequences were recovered from existing DNA constructs using PCR. The forward and reverse PCR primers included restriction enzyme sites (EcoR1 and Sal 1 repsectively) for insertion in frame with the correct orientation at the C-terminus of the MBP ORF.
Standard procedures were used for growing and inducing positive E.*coli*. The MBP of pMal-c2 vector accumulates in the cytoplasm of cells and is release by sonication. In a successful induction, MBP proteins accumulate to a large fraction of total soluble protein. We recovered proteins by affinity chromatography on amylose resin to which it binds and from which it is eluted with 10 mM maltose. We did not fully optimize induction conditions and yields were variable, but from each construct (for prox-ORF and Distal ORF fusions) we had at least one half litre culture that gave milligram amounts of protein, sufficient for our needs in analysis and in generation of additional immune regents. The elution of the prox-peptide from amylose resin is shown in Fig. 8 for the batch of protein we are using in most studies.
Purifed expressed proteins were quanitated by Bradford protein assay (BioRad) and also by western blot analysis with highly specific commercial antiserum to MBP (Fig. 9; this blot also provides a comparison for the anti-ORF peptide antisera of Task 1 above). Note that the much of the staining material larger than the MBP fusion in Fig. 8 also reacted with antiserum to MBP (Fig. 9) suggesting the apparent minor contaminants were either aggregates or possible translational read throughs that did not correctly recognize stop signals in the RNA.
The fusion proteins present the peptides to the immune system as a part of a larger protein that may be more antigenic than the peptides alone. We can screen mAb producing clones for immuoreactivity against the MBP, the fusion proteins and the available synthetic peptides in order to identify clones showing specificity for the proximal and distal promoter transcript uORF peptides.

Task 3: Comparative analysis of ER alternate promoter use and the expression of translation products
Year 1

Refine RT-PCR methodology for analysis of ER promoter use in small samples from sections

A one-step, RealTime RT-PCR method capable of quantifying absolute concentrations of the mRNAs transcribed from the distal and proximal promoters of the estrogen receptor in cell lines and tissues has been achieved. DNA templates specific for each transcript and flanking the real time RT-PCR target regions were prepared and quantitated by UV absorbance, these are used to generate standard curves (Fig. 10). The primer pairs for analysis of transcripts from alternate ER promoters are as we previously described (3). The modified assay allows direct separate estimation of transcripts from each promoter, rather than apportioning total ER mRNA level based on a ratio of alternate transcripts.

Standard curves were constructed for the assay of promoter use in breast tumors and cell lines. The uterine tumor line Ishikawa contained 0.022 and 0.012 amol/μg total RNA of the proximal and distal transcripts, respectively. The proximal to distal transcript ratio of 1.8 is in good agreement with previously determined ratios of approximately 3 by other assays (3). Consistent with our previous findings, the breast tumor line MCF-7 had no detectable distal promoter; the absolute proximal promoter concentration was 0.12 amol/μg of total RNA. In contrast, two estrogen receptor positive breast tumors had proximal/distal promoter concentrations of 0.0011/0.0003 and 0.0055/ 0.0013 amol/μg total RNA – ratios of 3.7 and 4.2, respectively. These data demonstrate that the very low concentrations of the estrogen receptor promoter transcripts can be quantitated by real time RT-PCR and suggest confirmation of previous studies indicating that the distal promoter transcript is more prevalent in resected breast tumors than in breast tumor cell lines and perhaps in noncancerous tissue where approximately equal concentrations of the two promoters reportedly exist.
The ability to quantitate alternate ER absolutely and with one tube for an assay is an improvement that will allow use of smaller tumor samples compared to our earlier technique. We can now run one experimental tube (for each promoter estimation in a sample) and obtain levels from a standard curve. In prior analyses we needed to run each sample with several levels of competitor to measure ER mRNA and then run an assay to determine the relative levels of the promoters.

KEY ACCOMPLISHMENTS

* Proof of principal that immune reagents can be developed to the peptides encoded in the uptream open reading frames of the alternate estrogen receptor transcripts

* Generation of uORF petides as fusions to maltose binding proteins for use as immunogens to generate monoclonal antisera

* Modification of RT-PCR assays for use in a RealTime format in the Roche LightCycler, development of assays to directly estimate alternate estrogen receptor promoter contribution to RNA levels.

REPORTABLE OUTCOMES

We have submitted, per contract requirements, an abstract to the Era of Hope meeting (fall 2002).

CONCLUSIONS

The funded work is executing the approved statement of work. Our studies address estimation of alternate Estrogen Receptor promoter use and should allow future determination of the relevance of alternate promoter use to breast cancer treatment and outcome. Our immune reagent approach is on track but remains high risk since we have no current evidence that the peptide targets are retained in cells after translation and the small peptides are difficult subjects for development of strong antibodies. The methodology for estimation of the promoter source of estrogen receptor from RNA has been improved and is now available for analysis of multiple samples in a research setting, though it would still be challenging to utilize in a clinical support environment.

RFERENCES

1. **Osborne CK** 1998 Steroid hormone receptors in breast cancer management. Breast Cancer Research & Treatment 51:227-238
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APPENDICES See attached figures (one through ten).

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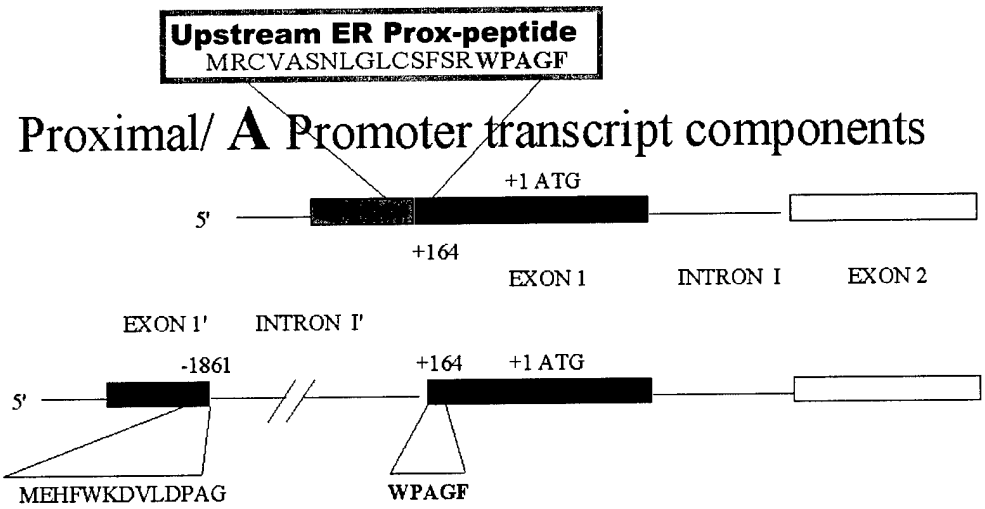


Fig. 1. Organization of the 5' regions of the human ER-alpha gene: Two promoters (distal [B, lower] and proximal [A, upper]) are present in the ER- gene and utilized in reproductive tissues. Alternative promoter transcripts converge at nt 164 of the mature mRNA (numbering based on proximal promoter transcript) and have a common ER coding region. Short ORF's are present in the upstream 5' region, these differ in the their N-terminal regions but share 5 C-terminal residues, by (RNA) splicing. Peptide sequences are shown

Region	DNA Sequence and Potential Translational Product
Distal 5' uORF	MetGluHisPheTrpLysAspValLeuAspProAlaGlyTrpProAlaGlyPheTer ..caagcccATGGAACATTTCTGGAAAGACGTTCTTGATCCAGCAGGGTGGCCCGCCGGTTTCTGAgcc..
Proximal 5' uORF	MetArgCysValAlaSerAsnLeuGlyLeuCysSerPheSerArgTrpProAlaGlyPheTe ..gcgggacATGCGCTGCGTCGCCTCTAACCTCGGGCTGTGCTCTTTTTCCAGGTGGCCCGCCGGTTTCTG

Fig 1B: Translational Start Regions of Estrogen Receptor Open Reading Frames
The DNA sequences and entire potential encoded peptides of alternate ER transcript upstream regions are shown together . The five **shared codons** (from a common exon) of the proximal and distal transcripts are shown **Bold**.

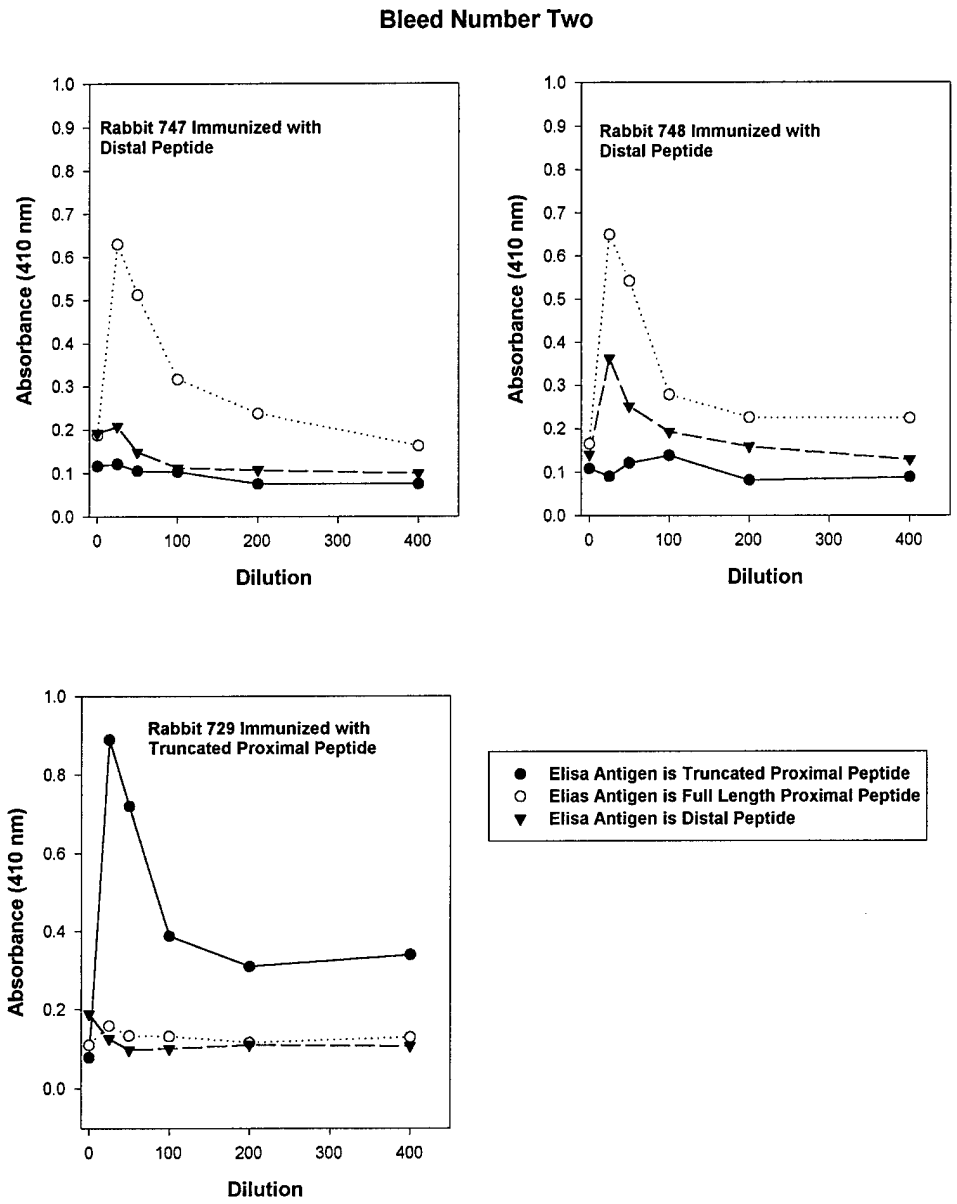


Fig 2: ELISA assay for test bleed of Rabbits after primary immunization and two boosts: Data for ELISAs on antisera from rabbits immunized with a full length synthetic peptide for the ER distal promoter transcript uORF peptide (#747 & #748) and one (#729) of the rabbits imm unized with the unique regions of the ER prox-uORF peptide. At this point ra bbit #747 antiserum is showing poor reactivity towards the distal peptide some reaction with the full length proximal peptide laid down in wells. Rabbit #748 is similar but showing a stronger reaction against the distal peptide. This suggest the major epitope in these antisera may include the common 5 residues absent from the shorter pr ox peptide. Antiserum from rabbit #729 showed strong reactivity against the immuni zing peptide (short unique form), but failed to react with the full length prox peptide (& the distal peptide).

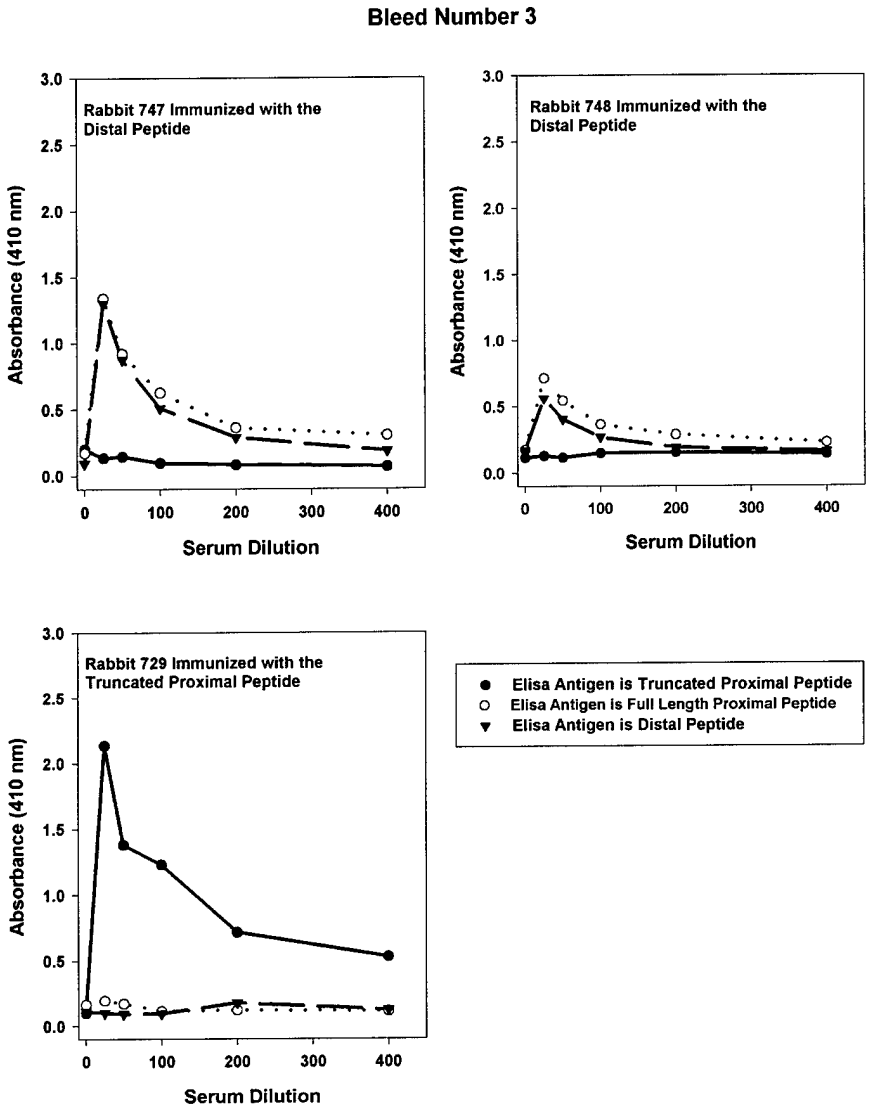


Fig 3: ELISA assay for test bleed of Rabbits after primary immunization and three boosts: Data for ELISAs on antisera from rabbits immunized with a full length synthetic peptide for the ER distal promoter transcript uORF peptide (#747 & #748) and one (#729) of the rabbits immunize d with the unique regions of the ER prox-uORF peptide. At this point rabbit #7 47 antiserum is showing a reactivity towards the distal peptide and the full length pr oximal peptide laid down in wells. Rabbit #748 is similar but weaker. This suggest the major epitope in these antisera may include the common 5 residues absent from the shorter prox p eptide. Antiserum from rabbit #729 showed strong reactivity against the immunizing peptide (short unique form), but failed to react with the full length prox peptide (& the distal peptide) indicating a blocking effect of the common C-terminal regions for the m ajor epitope recognized by serum #729.

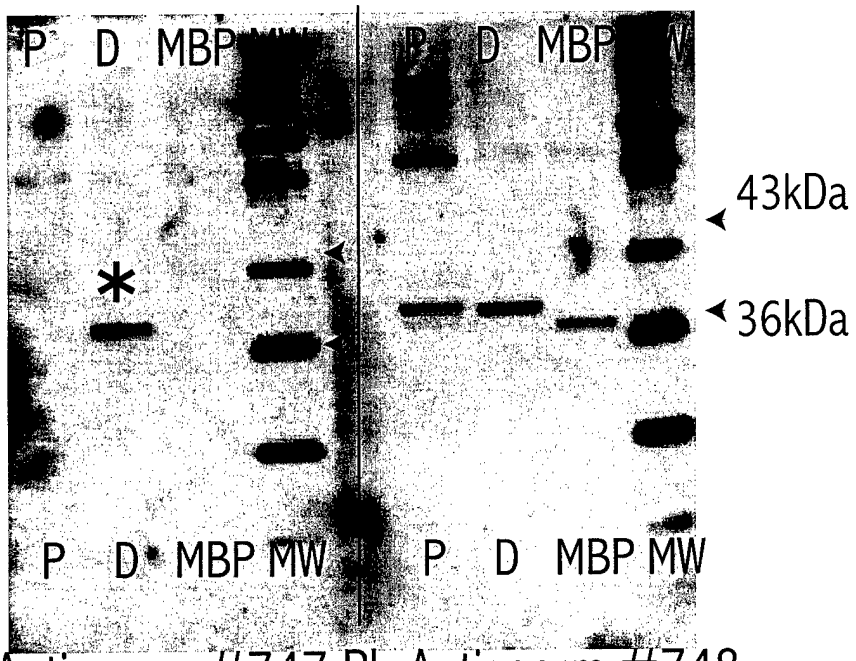


Fig. 4: Analysis of Rabbit antisera against the ER distal uORF peptide

Dilutions (1:200)of antisera from two rabbits (#747, 748) immunized with synthetic peptides corresponding to ER the distal promoter transcript uORF were tested in western immunoblots against 100ng purified E.coli expressed ~40kDa MBP proxpeptide -MBP (P) and distal peptide-MBP (D) c-terminal fusions. Samples analyzed in 10% acrylamide gels (NuPage) were blotted to immobilon. blots were incubated overnight with first Ab, bands in washed blots were detected using HRP-conjugated second Ab and Pierce supersignal for chemiluminescent detection. Only the #747 antiserum gave detection speicif for the distal peptide-MBP (*). The 748 antiserum cross rected with MPF and the prox fusion.

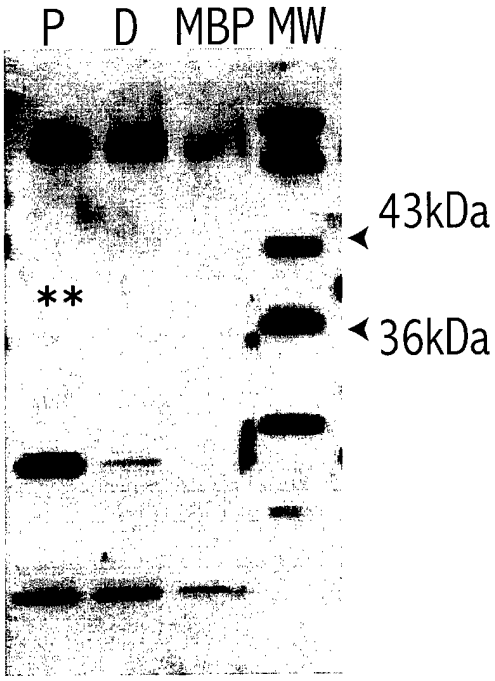


Fig. 5: Analysis of Rabbit antiserum against the ER prox uORF peptide

Dilutions (1:400)of antisera from rabbits #729, immunized with synthetic peptides corresponding to ER proximal promoter transcript uORF not shared with the distal was tested in a western immunoblot against 100ng of purified E.coli expressed ~40kDa MBP proxpeptide -MBP (P) and distal peptide-MBP (D) c-terminal fusions. Samples analyzed as described in Fig 4. At this dilution the antibody reacted with no MBP related proteins. No interaction was seen with the proxpeptide-MBP fusion (region **), suporting data from ELISAs. The antiserum of this rabbit dosea interact with low and high molecular weight proteins that appear to be from E.coli (this is quite common and is likely due to the rabbit having set up an immune response against gut flora).

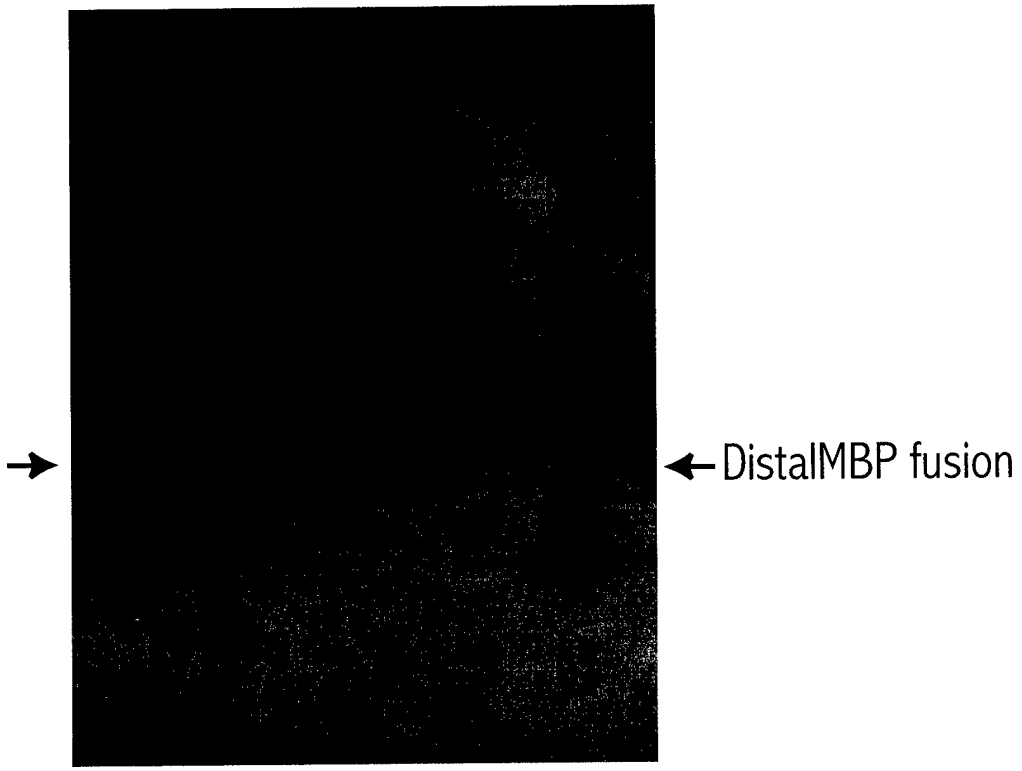
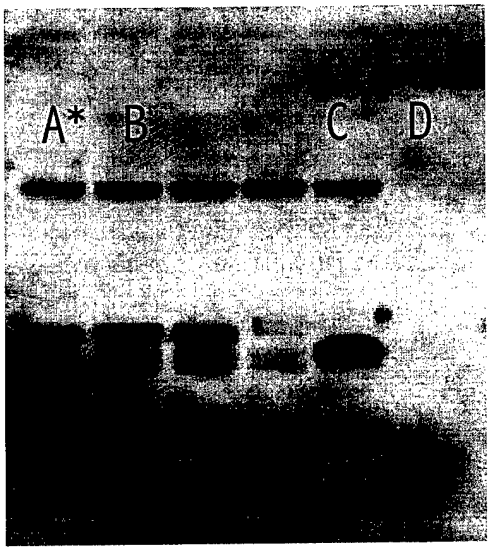


Fig. 6: Test for sensitivity of distal-MBP protein detection by #747 antiserum

A series of increasingly dilute samples of the distal-MBP fu sion protein were run on a 10% acrylamide gel, blotted and probed with the rabbit #747 antiserum rasied against the distal pro moter transcript uORF peptide. the limit of detection of the speci fic product was ~25ng fusion protein. This is for a 40KDa fusion protein, the effective concetnration of the distal peptide is ~1.25ng in this track (ie 2kDa out of 40kDA for the whole protein). We estimate a need to improve sensitvity ~10fold in order to have a reagent useful for use in cell and tissue studies.

Panel 1: GFP Ab



Panel 2: #747

Antiserum (distal peptide)

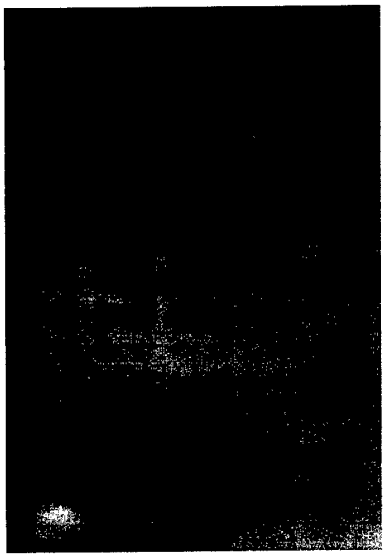


Fig. 7: Test of distal peptide Antiserum (rabbit #747)

Hela cells were transfected with a GFP-distal pepide fusion construct(A); a prox peptide GFP fusion (B) or parent vector pEGFPN1 (C). An mock rasnfected control HeLa was included (track D). Parallel blots were probed with antisera to GFP (Panel 1) or the distal ER upstream peptide (Panel 2, rabbit #747)

Transfected cells showed (panel 1) GFP or GFPfusion bands at ~20-22kDa and a refernce band at ~40kDa for a cotransfected reference that carries GFP fused to a 20KDa protein ORF. Note that track D is blank as there was no expressed GFP in track D.

Transfected and mock transfected cells showed similar patterns of bands in Panel 2, this is 'non-specific' staining of mammalian cell proteins. Track A (*) is the only track that should light up withe the #747 antiserum. Note that we can see no unique band around the 23kDa size region that is identified by one of molecular weight markers (MW).

The #747 antiserum was used at 1:200 and incubated with blots overnight. Samples were run in 10% acrylamide NuPage gels and blots were to immonbilon. Chemiluinescent detection with HRP- conjugated2nd Ab was used

Prox-peptide MBP fusion purification

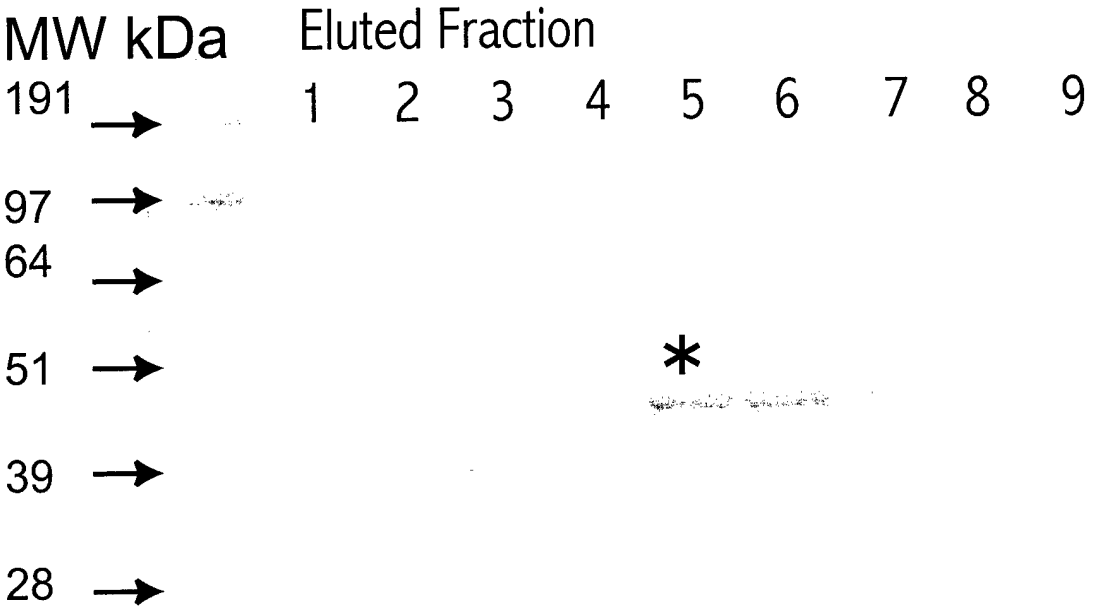


Fig. 8: One Step purification of an MBP-prox-peptide fusion protein using on Amylose resin
IPTG induced Ecoli carrying the pMal-c construct with a the prox peptide ORF as a c-terminal in frame fusion were lysed by sonication in buffered 200mM NaCl with 10mM Mecaptoethanol, and the lysate clarified by centrifugation. The supernatant was applied to an Amylose resin column (~7ml), washed with 10 voumes column and eluted with 10mM maltose in nine 1ml fracs. Aliquots of 5ul were analysed on 10% acrylamide gels. Frac5 was used to immunize mice and in Western blots.

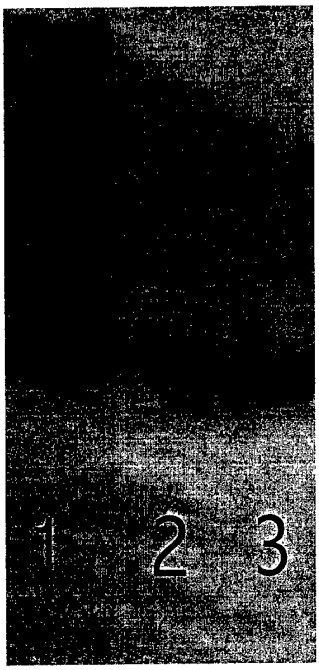


Fig. 9: Overexpression of uORF peptides as Maltose binding protein Fusions

Fusions proteins consisting of the ER uORF prox-peptide or the distal peptide linked to the C-terminus of the E.coli Maltose binding protein (MBP) were expressed in E.Coli and purifed on amylose columns (see Fig 8). Samples (100ng) of the prox-peptide MBP fusion (track 1), distal MBP fusion (track2) and MBP with no C-terminal fusion (track 3) were run in gels and western blots probed with antiserum to MBP. The fusions are larger than MBP alone due to the added peptides and because of additional several residues included as a buffer region after the MBP and prior to the peptides. Note that the high molecular weight protein seen in stained gels of the prox-peptide-MBP in Fig. 8 is immunoreactive and is therefore likely due to an aggregation effect or to translation through the fusion termination signal.These material was used to characterize rabbit antisera against uORF peptides in Task 1 and the fusion proteins are one form of the uORF peptides that have been administered to mice to generate monoclonal antisera (task 2).

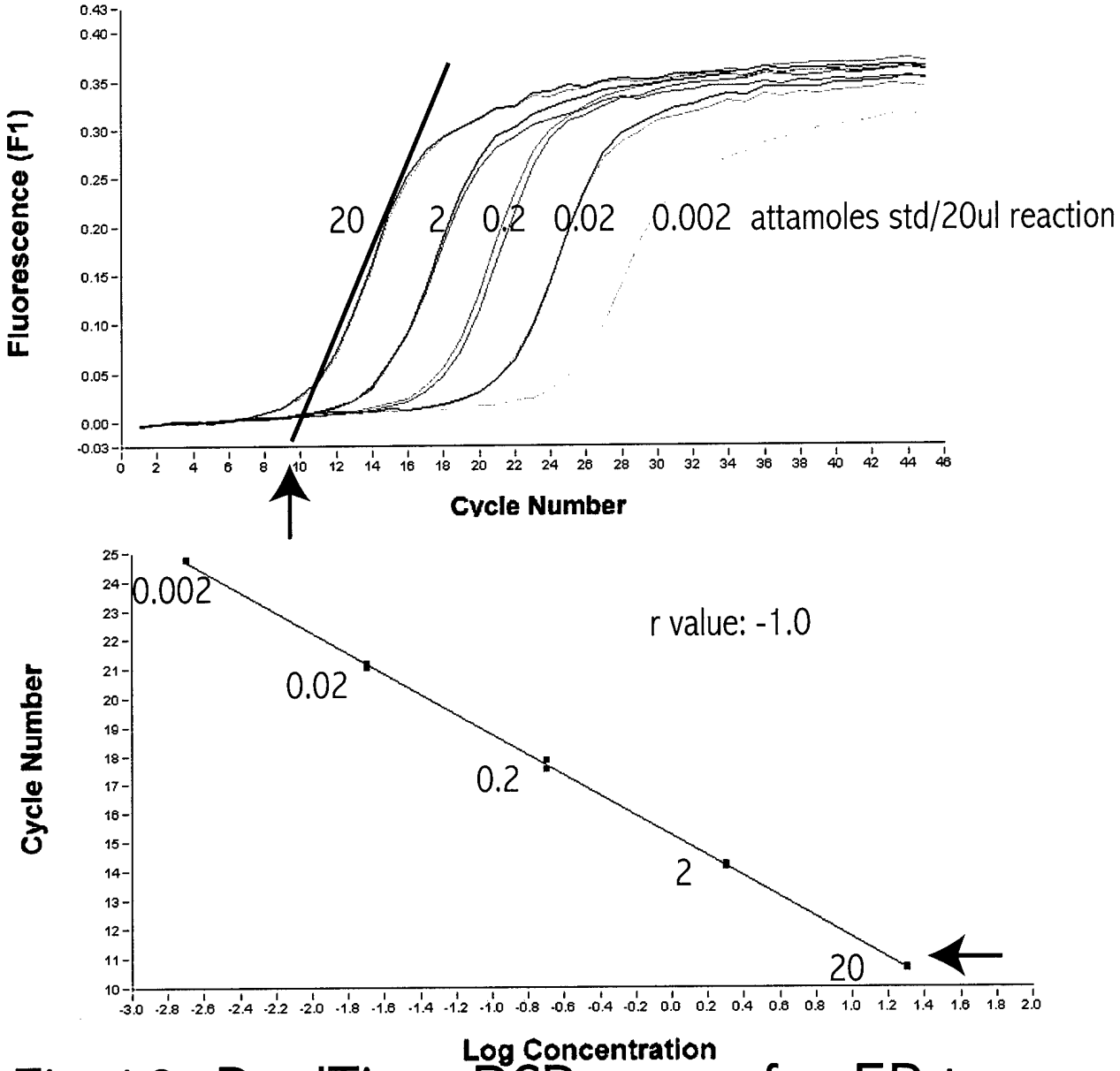


Fig 10: RealTime PCR assay for ER transcripts

Sample standard curve with distal ER promoter standard. The PCR reaction is performed with a series of dilutions in the Roche LightCycler. Fluorescence from DNA product is measured each cycle; data on concentration is estimated from extrapolation of the exponential phase slope. The high R value is a measure of th e robust nature of the technique. Data on samples (not shown) is determined in parallel RT PCR reactions that contain 100ng of RNA.