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This report describes the work conducted in the first year of a two year IDEA. Our original proposal was funded to study a human estrogen regulated transcript, HEM45, that encodes a novel protein of 181 amino-acid residues (M_r 20,300). The HEM45 protein has similarity to the bracket fungus protein FRT1 that can cause fruiting-body production and to a Xenopus product, XPMC2, that affects cell-cycle control. These similarities suggest that HEM45 has a role in mediating the estrogen action control of cellular proliferation and differentiation.
In the preliminary year of this project we have generated cell lines that over-express HEM45 mRNA in a controlled manner and antisera to both a peptide derived from the predicted HEM45 N-terminal region and to recombinant HEM45 protein. Preliminary characterization of the antisera demonstrates their utility in analysis of HEM45 protein expression and identifies a protein of the predicted mass in cellular extracts.

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FOREWORD

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Introduction:

The original research proposal described characterization of HEM45, a novel estrogen regulated gene identified by differential display polymerase chain reaction (PCR). That work (1) utilized a reconstructed, E_2-regulated system employing cells stably transfected with Estrogen Receptor (ER). The ER-transfected HeLa derivatives provide an ER-positive cell model with the availability of a powerful control in matched ER-negative lines.

Recent rapid expansion of the sequence databases due to development of sequence-tag strategies has aided rapid identification of the rat equivalent of human HEM45 during the period between submission of the original application and the beginning of funded studies. This allowed confirmation of the *in vivo* expression and estrogen regulation of the gene in-vivo in the uterus (1).

HEM45 is a novel gene; however, at the time of submission of the original proposal, the action of the related Xenopus XPMC2 sequence in *S. pombe* (2) and the capacity of the related Bracket Fungus Frt1 gene (3) to cause fruiting-body development suggested that HEM45 might have a role in mediating regulatory or developmental aspects of estrogen action. The Xenopus ovary XPMC2 cDNA sequence (2) can rescue mutant *S. pombe* from a mitotic catastrophe phenotype that was created by disruption of cdc2 kinase regulation by eliminating Wee 1 and Mik 1 kinase activities (4). Control of cdc2 kinase is a key step in controlling cell-cycle transition from G1 to S phase and G2 to M (5), but the point of XPMC2 action is unknown. XPMC2 does not cause the inhibitory tyrosine phosphorylation on cdc2 kinase (2), suggesting that it is not simply replacing Wee 1 and Mik 1 kinases in the rescued *S. Pombe* mutant. Frt1 from the basidiomycete *Schizophyllum commune* (Bracket Fungus) causes fruiting-body formation when introduced to suitable homokaryons, bypassing the need for fusion of two haploid strains of opposite mating types (3).

The N-terminal 9 to 30 region of the HEM45 ORF is relatively well conserved in the multiple sequence alignment, particularly with respect to XPMC2, suggesting a conserved structure/function for this region. Several mutations in the N-terminal region of *Frt1* abolished its capacity to cause fruiting-body development (3) and a potential dinucleotide binding motif is noted for the N-terminal region of *Frt1*. Limited homology to the gorilla CHPGOR sequence (encoding a nuclear autoantigen, 6) was also noted.

The Xenopus XPMC2 and Gorilla proteins are nuclear (2, 6) but the putative nuclear localization signals are outside of the regions aligning with Frt1 and the HEM45 ORF, making their cellular location problematic on the basis of sequence comparisons.

The HEM45 sequence was submitted to GENBANK (HSU88964, appendix item 1) in February and was released at the start of May 1997. At the end of May an essentially identical sequence (X89773, appendix item 1) from a European lab was revised and unembargoed almost two years after confidential submission. This sequence is apparently interferon induced in Daudi

transformed B cells, no further detail is yet available. Yeast and Caenorhabditis with significant protein level homology to the HEM45 ORF are now in the major databases (see BLAST search, appendix item 1). We intend to work with our biostatistics/ modeling colleagues to evaluate conservation of motifs within the HEM45 and to determine how the protein group has evolved, *e.g.* Whether separate yeast sequences represent analogs of the distinct primate HEM45 and related CHPGOR sequences. The existence of yeast analogues suggests that yeast genetics could be utilized to rapidly outline the role of the HEM45 family.

None of the additional, new, sequence data has linked us to genes for which a specific biochemical role has been unequivocally defined. Likewise, there has been no additional work published on the other family members that we defined in the proposal, hence the role of HEM45 still remains to be established.

One paper (1) on work relating to HEM45 has been accepted for publication. This work was largely undertaken on extensions of NIH R29 DK4706, but was prepared and submitted with the help of current support. The paper includes work included as preliminary data in the original grant proposal plus data on the rat HEM45 homolog that demonstrates expression/regulation of HEM45 *in vivo*.

The data available when the proposal was prepared led to development of two specific aims in a an IDEA format. These aims were centered around the hypothesis, based on homologies to FRT1 and XPMC2, that HEM45 might have a role in cell cycle control: In specific aim 1 we proposed to intervene in normal regulation of HEM45 by regulated over-expression of a transfected cDNA. In aim 2 We proposed to study the 'normal' expression of HEM45 by generating and utilizing antisera to the protein.

Body

Specific Aim I:

"We propose in **specific aim I** to over-express (independent of E_2/ER action) recombinant HEM45 protein (recHEM45) in normal and cancer derived mammary cells and HeLa cervical carcinoma cells. This should determine any HEM45 role in modulating cell growth and/or morphology. In addition we should be able to determine if the action of HEM45 protein is affected by the cell background. We shall compare HEM45 action in a tumor derived and 'normal' cell environments (MCF-7 & HeLa vs MCF-10A) and in cells from sites that usually gives rise to initially ER positive tumors as opposed to sites usually giving ER negative tumors (MCF-7/10A, Breast vs HeLa, Cervix)."

Task 1: months 1-3: Create cell derivatives carrying tetracycline-controlled trans-activator (tTa).
Task 2: months 4-6: Characterize tTa lines to allow selection of clones showing optimal regulated phenotype.
Task 3: months 1-5: Create expression constructs containing HEM45 sequences.

Task 4: months 5-7: Determine that HEM45 expression constructs are functional and regulated in transient transfection assays.
Task 5: months 6-10: Establish and clone out stable mammalian tTa lines carrying stably transfected HEM45 Tet regulated constructs.
Task 6: months 11-14: Evaluate clones, conduct preliminary studies on regulation of growth/morphology. Select candidate HeLa, MCF-7, MCF-10 derivative clones.

We chose to utilize a tetracyline (Tet) regulated system (7) (TET-ON) in which Tet analogue doxycyline (Dox) causes gene expression to be increased (activates the transactivator, tTa) (8), in contrast to earlier version of the Tet regulated system (discussed in the proposal), where continuous application of antibiotic is required to suppress gene expression. We were able to obtain a HeLa cell host (Clontech) and we have created suitable MCF-7 lines (ER positive, breast tumor line) expressing the modified, activatable, tTa from a transfected construct. The MCF 7 lines may be of general utility in the scientific community. We utilized a low passage MCF7 (ca passage 180) from American Type Culture Collection to reduce the risk of 'drift' in cell characteristics, but the downside of this was a long generation time for the cells. These 'TET-ON' cell lines must be transfected with the sequence of interest under the control of a tTa regulated promoter.

Both the Hela and MCF-7 derived lines have been successfully transfected with expression constructs (tTA regulated pTRE with HEM45 open reading frame (ORF) insert), and sublines isolated which demonstrate doxycyline inducible expression of HEM45 mRNA by PCR analysis (Figs. 1 and 2). These studies are the work of Dr K Bove, a research associate supported by the grant/contract. An immortalized, non-tumorigenic line fitted with the Tet-regulated system has not been obtained. We have attempted to introduce the Tet-regulatory system to both 'normal' MCF10A (spontaneously immortalized breast cell line) line as originally proposed and to normal 184A breast cell line (benzpyrene treated to achieve immortalization). Clones of an aggressive breast cancer line lacking ER, MDA-MB-231, that harbor the Tet-regulatory system are under analysis, and provide an additional phenotype (breast tumor line, ER negative) that was not part of the proposal. Dr Pentecost expended the effort to isolate these extra lines so as to have a matched set of tumor lines both for the current studies and other purposes. Candidate MDA-MB-231 derivatives will be available for transfection with the regulated HEM45 constructs sequences.

Dr Pentecost recently undertook a brief study of HEM45 expression in a range of breast cell lines representing phenotypes from non-tumorigenic to hormonal responsive to hormone independent. The data are relevant to the funded activity, though not part of the original proposal. High levels of expression (relative to MCF-7) were seen in both a non-tumorigenic (MCF10A) and a highly aggressive breast cancer line (MDA-MB-436) (Fig. 3). The range cell lines were from a panel utilized by Dr David Spink of Wadsworth Center as part of studies of regulation of estrogen metabolizing P450s, a project in which Dr Pentecost has a minor role. These data suggest that one concept behind the original application, which was implicit and not well developed, is incorrect: HEM45 mRNA expression obviously does not directly or inversely correlate with the

tumorigenic/metastatic status of breast derived cell lines. However, the explicit aim, to study the role of HEM45 in cell control, remains valid. We cannot be sure, without further experiments, that the high levels of HEM45 mRNA in the two lines are not simply a reflection of the growth state they were in at the time of harvesting or some other factor which we have not fully controlled. However earlier data, include in the original proposal, showed not effect of cell density, *per se*, on HEM45 mRNA levels in UP1 ER positive HeLa derived cells.

In figures 1 and 2 we present preliminary analysis of Dox-regulated expression of HEM45 from the pTRE vector. This analysis was by PCR (as summarized in figure legends) and utilized a primer set that contained one primer that anneals to sequences with HEM45, endogenous or transfected, and a reverse primer that is specific to 3'-untranslated sequence of the HEM45/pTRE construct *i.e.* is not to HEM45 but to associated vector sequences that provide a poly-adenylation signal etc. (Fig. 1.A).

Analysis of recHEM45 mRNA in two HeLa derived lines clearly show Dox dependent induction of a ~600 bp product (Fig. 1.B) - the predicted size of the PCR product from the HEM45 chimeras. The Hela lines were created in a commercially available 'TET-ON' HeLa derivative. A major limitation of the Tet regulated system is the need to evaluate multiple candidate lines at each stage of creation. The two HeLa derived lines used in Fig. 1 exhibit the best regulation and induction out of over 12 lines created and tested. Figure 2 shows similar results for an MCF-7 derived line that we created. As described in the original plan and statement of work, this required isolation of multiple candidate regulator (tTa) transfected MCF-7 lines under G418/neomycin selection. These were evaluated by transient transfection with a pTRE-luciferase construct whose regulation could be easily monitored using a luminescence spectrophotometer for analysis of luciferase in cell extracts. A good line with strong inducibility was then stabley transfected with the pTRE-HEM45 construct (using co-selection for puromycin resistance) and the KB.M5DS-36 'double stable' line identified. In the presented data (Fig. 2) we have probed a blot of the PCR product for HEM45 as the MCF-7 samples show a heavy non-specific PCR background. Clear induction of the HEM45 chimera is seen following 48 h treatment with 0.5 and 2 μg/ml antibiotic, as compared to the no-drug control. For comparison we demonstrated that the common reference sequence Glyceraldehyde phosphate Dehydrogenase (GA3PD) was constant in cells undergoing these treatments.

The HeLa and /MCF-7 lines expressing inducible recHEM45 mRNA are now available for analysis in task 7, evaluation of effects of altering HEM45 expression (see future direction).

Specific Aim II:

"In **specific aim 2** we intend to determine the intracellular localization of the HEM45 as an additional means of addressing its cellular function/role."

Task 8: Months 1-6: Obtain peptides, raise anti-peptide antisera in rabbits.
Task 9: months 7-12: Characterize antisera, purify if necessary, do initial cell experiments.

Task 10: months 1-6: over-express HEM45 protein in E.coli by making pET constructs.
Task 11: month 7-12: purify recHEM45, immunize rabbits.
Task 12: months 13-15: characterize recHEM45 antisera, immunopurify if necessary.

The work of aim 2 scheduled in tasks 8-12 has been achieved. This puts this part of the project ahead of schedule and has resulted in preliminary data which may lead to modification of aim I. The progress of aim 2 has been facilitated by the effort of additional State personnel: Dr James Dias (named as a collaborator in the proposal) and Dr Andrew Peterson, who has done much of the lab work in generating recHEM45 and characterizing the Antisera to both the peptides and recHEM45. Preliminary work on task 13 (Do detailed immunohistochemical studies with antisera) has begun.

Expression of Recombinant HEM45 protein

cDNA to HEM45 mRNA (1) was made using reverse transcriptase and the HEM45 open reading frame (ORF) amplified by polymerase chain reaction (PCR) with primers which permitted in-frame insertion into the expression vector pET-15b(9) (Novagen, Milwaukee,WI) due inclusion of suitable restriction sites. The pET-15b vector allows controlled expression in E. coli strains carrying inducible T7 DNA polymerase genes (10). The fusion protein formed by insertion of the gene of interest into the vector also carries a leader with both a poly Histidine motif, permitting rapid partial purification by Nickel-ion chelation chromatography (11), and a thrombin cleavage site allowing subsequent removal of most of the leader sequence. Recombinant HEM45 was expressed in *E.coli* transformed with the expression vector pET-15b. Four hours after induction with 2 mM IPTG, cells were harvested. Inclusion bodies containing insoluble HEM45 protein were enriched by resuspending cells in cold 5 mM imidazole, 500 mM NaCl, 20 mM Tris pH 7.9 buffer, and sonicating the suspension three times to release soluble protein. Inclusion bodies were harvestedas a pellet by centrifugation at 20,000 x g for 15 minutes. The pellet was washed two additional times as described. After the final wash, inclusion bodies were solubilized by resuspending the pellet in cold 8 M urea, 5 mM imidazole, 500 mM NaCl, 20 mM Tris pH 7.9. The resuspension was sonicated and kept at 4°C. The suspension was clarified by centrifugation at 25,000 rpm for 20 minutes in a SW55 Ti rotor. The supernatant containing amine terminal His_6-tagged recombinant HEM45 protein was passed over a Ni^{2+} His-bind resin column (Novagen, Milwaukee, WI). The affinity column was washed with 8 M urea, 20 mM imidazole, 500 mM NaCl, 20 mM Tris pH 7.9 and the HEM-45 eluted with 8 M urea, 300 mM imidazole, 500 mM NaCl, 20 mM Tris pH7.9. HEM45 was purified from the column-eluted material on preparative SDS-15% polyacrylamide gels, slicing the polypeptide band from the gels, and electro-eluting the HEM45 protein.

Production of HEM45 Antisera

Flemish giant-chinchilla cross rabbits were immunized with gel-purified recombinant HEM45 protein or a synthetic peptide corresponding to HEM45 sequence 3-16 (^{3}GSREVVAMDCEMVG^{16}) as predicted from the HEM45 cDNA (1). The synthetic peptide was coupled to ovalbumin through an engineered carboxyl terminal cysteine (12). Rabbits were immunized four times, bi-weekly, with one milligram peptide equivalents at multiple dorsal

sites. Initial immunization was in complete Freund's adjuvant, subsequent immunization was in incomplete Freunds. Use of animals was approved by the Wadsworth center IACUC and is an approved part of the funded research project. Serum collected after the fourth immunization was cleared by caprylic acid and ammonium sulfate precipitation (13).

Immunoblot Analysis.

HeLa cells were removed from culture flasks by washing with a solution of EDTA. Harvested cells were counted, pelleted, and solubilized in electrophoresis sample buffer. Samples were boiled for 5 minutes before electrophoresis on SDS/15% polyacrylamide gels (14). PAGE resolved polypeptides were electro-blotted for 1 h at 0.5 amps in Towbin's buffer onto Immobilon membrane (Millipore, Bedford,MA)(15). Protein blots were probed with HEM45 antibodies, and then processed by for ECL Western blot detection (Amersham, Arlington Heights, IL). Results from western blot analysis were quantitated with an Image Master Scanner/Densitometer (Phamacia, Piscataway, NJ).
Task 7: months 15-22: Conduct studies on effects of HEM45 regulated expression on growth and morphology. Include immunohistochemical studies using HEM45 antisera.

Characterization of recombinant HEM45 protein

HEM45 was expressed in bacteria as a fusion protein, the 181 amino acid HEM45 polypeptide and 20 amino acid amine-terminal leader containing 6 histidine residues. The oligohistidine amine terminal leader allowed partial purification of recombinant HEM45 using metal chelation chromatography. The amine terminal leader possessed a thrombin cleavage site which allowed removal of seventeen of the twenty amino acid leader. As shown in figure 4A, digestion of recombinant HEM45 with thrombin produced a polypeptide of approximately 20,500 Da, as predicted by HEM45 cDNA (1). To verify further, the authenticity of bacterially expressed protein as HEM45, thrombin digested protein was subjected to immunoblot analysis using an antipeptide antisera against HEM45 peptide sequence 3-16. Both thrombin digested and undigested recombinant HEM45 protein were found to immunoreactive (Fig.4B). These data confirmed that we had: 1. Successfully expressed recombinant HEM45 protein in bacteria and 2. Produced an anti-peptide antisera reacted with HEM45. Recombinant HEM45 protein was further purified on SDS-polyacrylamide gels, isolated and used as an immunogen. Figure 5A.2 shows that antibody made against recombinant HEM45 elicits the same immunological signal on immunoblots as anti-peptide antisera against HEM45 peptide sequence 3-16 (Fig 5A.1). Both antisera recognized a 20 KDa polypeptide in HeLa cell lysates (Fig. 5).
It is clear (Fig.5) that the antibody against recHEM45 gives fewer 'extra' bands than the anti-peptide antiserum when used with eucaryotic cells. However, the cross-reaction of the anti-peptide antiserum can be blocked by ovalbumin (not shown) - indicating that the components cross reacting are against the ovalbumin to which the peptide was couple for immunization.

Characterization of native HEM45 in Proliferating Cell Cultures
In preliminary characterization of antisera we measured HEM45 protein levels in HeLa cell monolayers at various stages of confluence by western immunoblot analysis. In this study we utilized the antisera against the HEM45 3-16 peptide, demonstrating that it could detect a ca 20 K Da protein. Figure 6 demonstrated that the steady state level of HEM45 increased in a linear fashion (0-18 h) with time. Cell confluence was reached at the 18 h time-point. Immunoblot analysis of culture media from cell monolayers, found HEM45 not to be secreted from cells (Data not shown)..

Immunohistochemistry for HEM45 in Hela
Preliminary immunohistochemistry (using peroxidase staining, Vector labs) with X826 antipeptide antisera to the HEM45 N-terminal peptide (Fig. 7) in sparse cultures indicates that HEM45 is in or around the nucleus, but this is under detailed analysis using the antisera against the recombinant protein and more sophisticated imaging techniques. By immunohistochemistry, the levels of detectable HEM45 fall dramatically as cell reach confluence (not shown). Paradoxically, this is the reverse of what was seen with the Western of Fig. 6. The conflict between this data and that for analysis by Western blot (Fig. 6) could relate to redistribution of HEM45 within the cell (an equivalent amount of protein in the cytoplasm is more difficult to detect than the same amount of protein on or in the nucleus). Alternatively, and the explanation currently being evaluated: the cell density experiments using western blots (Fig. 6) utilized cells that came from very confluent stocks and the increase in HEM45 with time may reflect reaccumulation of HEM45 following replating at subconfluence, rather than any increase in cell density in the period following replating.

Future Direction:
A number of 'reagents' have been developed in the first year of this two year project: 1. Tumor cell lines have been developed that carry systems allowing the regulated overexpression of HEM45 mRNA, 2. Antisera have been developed to HEM45 that can recognize both the denatured protein on nitrocellulose and the protein in fixed cells. The goal of the remaining year of the project is to utilize these resources to understand the regulation and role of HEM45. This is formally tasks 7 and 13 of the Statement of work. We have not yet generated a 'TET-ON' variant of an immortalized, non-tumorigenic, breast cell line.

Aim I: We had originally planned to prepare all the HEM45 transfected cell lines and then study effects on cell growth etc. We now feel, in light of difficultly in obtaining transfected non-tumorigenic lines that we need to begin studying cell effects with available lines. In addition the data of Fig. 3 suggest that HEM45 mRNA expression does not correlate with the cancer phenotype. A better understanding of the role of HEM45 will allow us to reevaluate the need for the MCF10A derivative.

We were surprised at the relative ease of detecting HEM45 in blots and cells. The data of aim 2 indicates that there is 'more' HEM45 protein in 'control' cells than we expected from the low basal level of HEM45 mRNA in the original battery of cells evaluated. The PI has therefore

worked to create lines of tTa transfected cells (from the parent lines purchased or created by Dr. Kathleen Bove) carrying Dox regulatable anti-sense constructs for HEM45. Several cell lines have been cloned, but non have been found to express detectable antisense HEM45, though it is not clear if this is a problem of detection or of the cells being negative for antisense expression. This new approach continues, and posits that ablation of HEM45 mRNA may better alter HEM45 protein levels than attempts at over-expression, and hence provide a better means to understand any influence of HEM45 on cell properties.

We need to optimize Dox treatment of the HEM45 transfected 'TET-ON' cells, with regard to time and concentration before moving on to study effect on 'cell growth and morphology'. We have to determine how expression of HEM45 mRNA from the Tet regulated system affects net HEM45 protein in the cell, and/or determine if we can perturb the patterns of HEM45 protein expression seen in non-transfected cells (eg see possible effects of cell density on HEM45 expression, fig. 6).

Our approaches to analysis of cell growth in the original proposal were not well defined. Tissue culture well based cell/DNA/Protein counts, are feasible -as proposed in the original application. However, homologies suggest a potential role for HEM45 in cell cycle control and there are more direct ways of evaluating cell cycle behavior: We have recently come to appreciate the power of flow cytometry in analysis of cell cycle. This seems applicable in monitoring cell cycle progression following manipulation of HEM45 mRNA expression in the TET-ON cells.

Methodology has been established for cell cycle monitoring by flow cytometry. The ethanol fixation/permeabilisation approach (16) proved more effective than a method using triton lysis of paraformaldehyde fixed cells. Very preliminary experiments (utilizing the TET ON HeLa carrying inducible recHEM45 constructs) did not indicate an effect of Dox treatment on the distribution of cells in the phases of the cell cycle. However, as noted above, we still need to optimize induction time and antibiotic level and to examine effect of recHEM45 mRNA induction on overall HEM45 protein expression.

The preliminary studies were done with unsynchronized cultures. Future experiments will include synchronization of cells and analysis of cell cycle progression on release, or ability to overcome the blockade as a result of HEM45 over-expression. We also have the resources to directly and simultaneously monitor cells cycle progression (eg. using the available UV laser and Hoescht dye in viable cells, or using the argon laser with propidium iodide in permeablized cells) while monitoring intracellular HEM45 using FITC tagged antibody. HEM45 is an intracellular protein, not a cell surface protein, hence cell permeablisation would be required, either in fixed cells or by generation of transient pores (eg with saponin).

Aim 2: Studies are ongoing to determine the subcellular localisation of HEM45 by fluorescence and confocal microscopy. If appropriate, these studies can be extended to Electron Microscopic approaches.

Studies of protein expression may be extended to analysis of protein in cells subjected to synchronization, such studies will complement the work of Aim 1. In integrating the themes of the two aims, we need to evaluate the expression of HEM45 protein in normal cells in addition to tumor cells, as immortalized, non-tumorigenic cells are distinct in having a true resting state. Estrogen induction of HEM45 protein also must be addressed in ER positive settings, though with care to avoid confusing direct effects with indirect effects related to estrogen affecting growth and hence cell density.

Conclusions:

Our original proposal was funded to study a human estrogen regulated transcript, HEM45 that encodes a novel protein of 181 amino-acid residues (M_r 20,300). The HEM45 protein has similarity to the bracket fungus protein FRT1 that can cause fruiting-body production and to a Xenopus product, XPMC2, that affects cell-cycle control. These similarities suggest that HEM45 has a role in mediating the estrogen action control of cellular proliferation and differentiation.

In the preliminary year of this project we have successfully generated tumor cell lines that over express HEM45 mRNA in a controlled manner and antisera to both a peptide derived from the HEM45 ORF and to recHEM45 protein. Preliminary characterization of the antisera demonstrates their utility in analysis of HEM45 protein expression and identifies a protein of the predicted mass in cellular extracts. We are now positioned to actively work on defining the role of HEM45 in the time remaining for the project.

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16. Crissman, H.A., Steinkamp J.A., (1973) Rapid, simultaneous measurement of DNA, protein and cell volume in single cells from large mammalian cell populations. *J Cell Biol* **59**, 766-771.

NCBI Entrez --Nucleotide QUERY BLAST Entrez ?

Other Formats: FASTA Graphic
Links: Protein Related Sequences

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  AUTHORS   Brian T Pentecost.
  TITLE     Expression and Estrogen Regulation of the Novel HEM45 Sequence in
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  JOURNAL   Unpublished
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  AUTHORS   Pentecost,B.T.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-1997) Laboratory of Reproductive and Metabolic
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NCBI Entrez Protein QUERY BLAST Entrez ?

Other Formats: FASTA Graphic
Links: DNA Related Sequences

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  AUTHORS   Tissot,C., Nissen,J. and Mechti,N.
  TITLE     Molecular cloning of a new interferon-inductible PML nuclear
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  JOURNAL   Unpublished
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  AUTHORS   Mechti,N.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-JUL-1995) N. Mechti, Institut de Genetique
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NCBI **BLAST Search Results** Entrez ?

BLASTP 1.4.9MP [26-March-1996] [Build 14:27:01 Apr 1 1996]

Reference: Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J. Lipman (1990). Basic local alignment search tool. J. Mol. Biol. 215:403-10.

Query= gi|2062680 HEM45
(181 letters)

Database: Non-redundant GenBank CDS translations+PDB+SwissProt+SPupdate+PIR
276,695 sequences; 78,740,169 total letters.

Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)	N
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pir||S53818	XPMC2 protein - African clawed frog ...	190	5.1e-30	3
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pir||S55252	mushroom-inducing protein Frt1 - bra...	84	2.9e-09	3
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pir||S64944	hypothetical protein YLR107w - yeast...	54	0.00068	4
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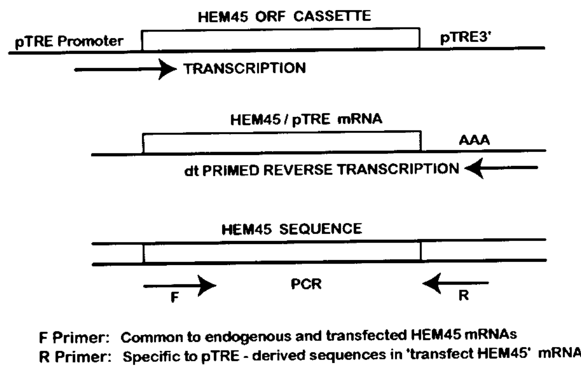
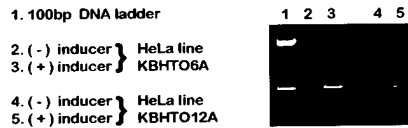
A. Strategy for HEM45 Transfect - Specific Analysis



B. Product of pTRE - HEM45 Expression in HeLa Lines



'Tet - Regulated' HEM45 Chimeras: Expression of mRNa

Figure 1: Analysis of recHEM45 mRNA in HeLa cells by 'allele specific' RT-PCR
Cloned HeLa derived lines were created that express recHEM45 mRNA under the control of an inducible promoter in pTRE (8). **Panel A** summarizes the strategy to analyze cell RNA for the HEM45 generated from the expression construct. RNA was prepared from cell lines using Trizol reagent (Life Technologies, Gaithersburg, MD). First strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (MMLV-RT). Equal samples of each RT reaction were subjected to the polymerase Chain Reaction (PCR) procedure in a GeneAmp PCR System 9600 using the following cycling parameters: 94°-180s, Taq polymerase added (hot start), 25x[94°-30s, 60°-15s, 72°-30s]. The forward primer (5'-^{61}GCCATGGACTCCGAGA^{77}-3') was to human HEM-45 . The reverse primer (5' -^{541}CACTGCATTCTAGTTGTGG^{523} -3') was to a sequence in the Bujard and Gossen Tet-regulated plasmid, pTRE (7) that forms part of the chimeric mRNA. Thus these primers should exponentially amplify only HEM-45 of the transfected pTRE-HEM45 construct.
Panel B shows the induction of recHEM45 mRNA in two selected lines following treatment with Doxycyline (1 μg/ml, 48 h). Equivalent samples of all PCR products were analyzed by agarose electrophoresis and DNA products visualized by UV illumination of ethidium bromide. The PCR product, at slightly greater than 600 bp (bright band in 100bp ladder), was of the predicted size. These cloned lines are 'double stable transfects', having first been transfected (by Clontech) with the 'Tet regulatory system' (G418 selection for neomycin resistance) and then with the pTRE-HEM45 construct (co-selection for puromycin resistance construct pUR).

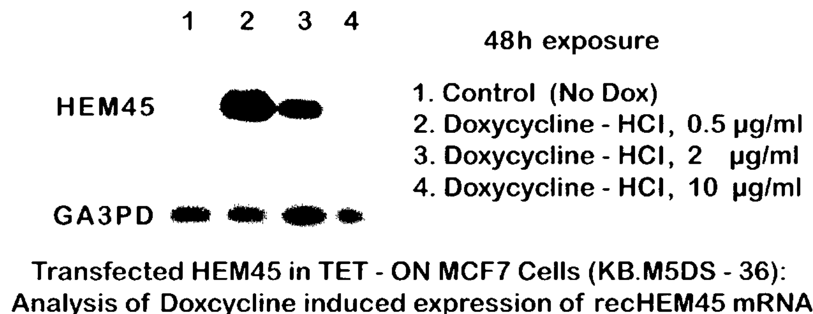
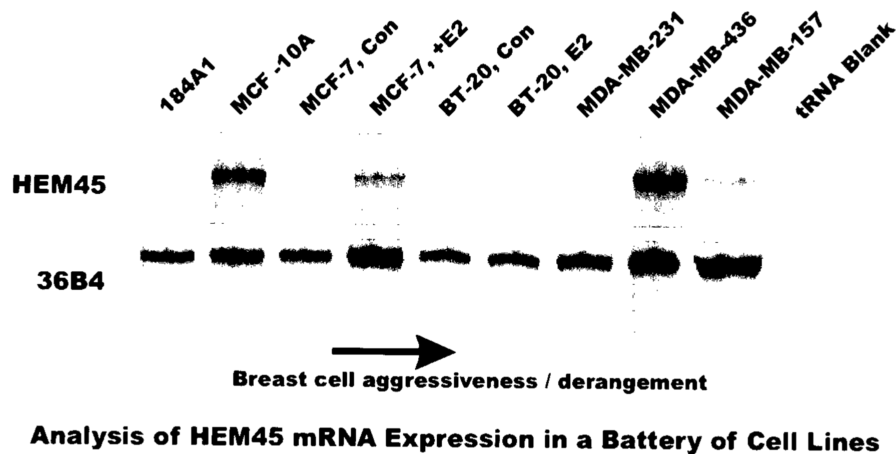


Figure 2: Analysis of recHEM45 mRNA in MCF-7 cells by 'allele specific' RT-PCR

Clear regulation of recHEM45 mRNA was demonstrated at 0.5-2 µg/ml Doxycyline in a cell line created from MCF-7 breast cancer cells. MCF-7 derived line KB.DSM5-36 carrying both the Tet regulator and the HEM45 ORF in regulated construct pTRE was created by transfection of low passage (ca 180) MCF-7 cells. Sequential transfection and cloning first introduced the Tet-responsive regulator and then the expression plasmid (see Fig.1 description also). Cells were plated out in T 25 flasks (1:4 split) and grown in DC5 media (phenol red-free Dulbecco's Modified Eagle's Medium supplemented with L-glutamine (1%), non-essential amino acids (1%) and bovine calf serum (BCS, 5%)) until treatment 2 days later, cells were then switched to stripped serum DC5 (8ml) and treated with Doxycyline at the indicated levels (0, 0.5, 2, 10 µg/ml). Scheduling was designed to avoid overgrowth of cells prior to the end of the 48 h treatment period. RNA was prepared using Trizol reagent.

cDNAs were synthesized from total RNA template using dT primer and MMLV-RT (see Fig.1.) Reverse transcript from 0.2 µg input RNA was subjected to PCR using a step-down protocol (96° 300s, 3x[96°-30s, 68°-30s, 72°-60s], 3x[96°-30s, 64°-30s, 72°-60s], 25x[96°-30s, 60°-30s, 72°-60s]) in an Ericomp thermocycler. The same primer set as used in Fig. 1 was employed for analysis of recHEM45 mRNA expression in the MCF-7 derived line. Parallel PCR of GA3PD provided an indication that the RT reaction was successful for all samples, and gave a ~100 bp product. Fragments in the entirety of each reaction were resolved in agarose gels, and probed with 32P labeled cDNA fragments after southern blotting. Blotting and probing was employed due to presence of additional non-specific bands in PCR samples. Image data was acquired by suitable periods of autoradiography on XAR film.



Analysis of HEM45 mRNA Expression in a Battery of Cell Lines

Figure 3: Analysis, by RPA of HEM45 mRNA expression in Breast Cell lines

Non-tumorigenic MCF-10A and 184-A1 lines (Estrogen Receptor negative) were maintained in DMEM/F12 supplemented as described in text. Other cell lines were maintained in DC 5 Medium. MCF-7 and BT-20 were grown in DC 5 medium and switched to sDC 5 medium on the day prior to estrogen treatment, estrogen exposure was for 6 h. Estradiol was added to cells at 10 nM as a 1:1000 dilution of ethanol stocks, the control cultures received vehicle alone. MCF-7 is a classically ER positive cell line, were we have previously shown HEM45 to be estrogen responsive (1), BT-20 has, in our hands (Spink *et al* submitted) a very low level of ER mRNA. The MDA series lines represent aggressive tumor lines lacking ER. RNA was prepared from cultures using Trizol reagent. Analysis of HEM45 in cell lines was by RNA protection assay utilizing HEM45 and 36B4 reference probes as described. The HEM45 mRNA protects a probe fragment of ca.82nt. 5 µg of total cellular RNA was include in the hybridization step of the assay. Methodology was as described (1) (see appendix item 3).

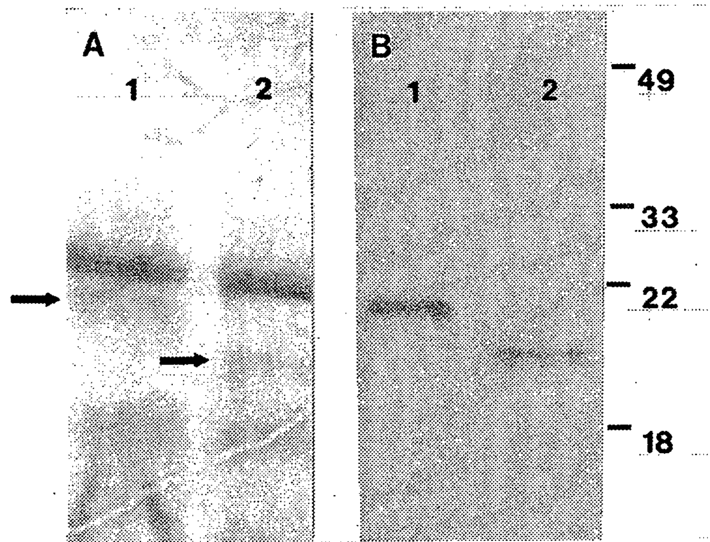


Figure 4: Characterization of recombinant HEM45 by immunoblotting using an antipeptide antisera against HEM45 peptide sequence 3-16.

Silver stained gel (A) and Western immunoblot (B) of partially purified recombinant HEM45. 5 ug of recombinant HEM45 was digested with 1.5 u of thrombin for 30 minutes and electrophoresed into 15% SDS-polyacrylamide gel under reducing conditions, half the gel was transferred to Immobilon P membrane, and challenged with rabbit antipeptide antisera X-826 against HEM45 peptide sequence 3-16 (1:500 dilution). The secondary antibody was donkey anti-rabbit IgG-alkaline phosphatase (1:2,000 dilution), and developed with Western Blue substrate (Promega, Madison, WI). Lanes: 1. undigested HEM45; 2. thrombin digested HEM45. The relative migration positions and size (x 1,000 Dalton) of molecular weight markers are noted at the right of panel. Position of the putative HEM45 bands in panel A is indicated by arrows.

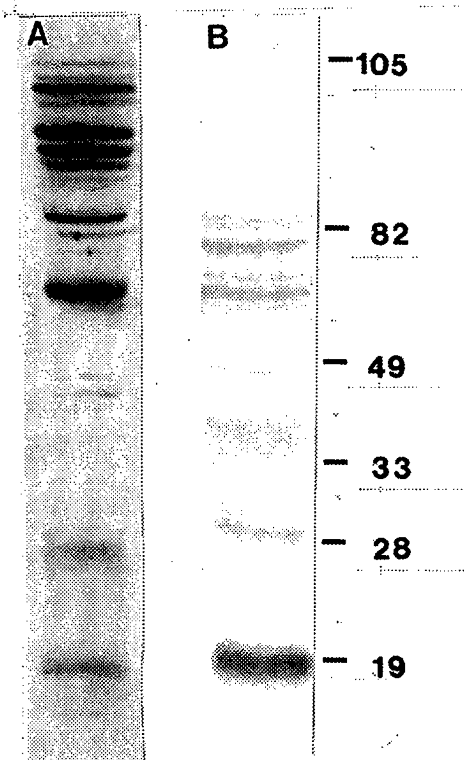


Figure 5: Detection of HEM45 by western immunoblot analysis of a HeLa cell lysate.

Lysates prepared from a HeLa cell culture ($2x10^5$ cells/lane) were electrophoresed into 15% SDS-polyacrylamide gel under reducing conditions, transferred to Immobilon P membrane, and challenged with either rabbit anti-peptide antisera against HEM45 peptide sequence 3-16 (A)(1:500 dilution) or rabbit Y29 antibody (B) (1:500 dilution). Antisera Y29 was prepared by immunizing rabbits with gel purified recombinant HEM45 (please see Fig. 4.A). The secondary antibody was donkey anti-rabbit IgG-alkaline phosphatase (1:2,000 dilution). Blots were developed for ECL detection (Amersham). The relative migration positions of molecular weight markers are noted at the right of the panel (x 1,000 Dalton). Both antisera detected a band of the predicted size (~20 000 Da)The anti-rec HEM45 antiserum Y29 gave a lower degree of cross-reaction than the anti-peptide antiserum. However, the cross-reacting bands of the anti-peptide anti-serum can be largely blocked by pre-incubation of antiserum with immunization carrier protein ovalbumin.

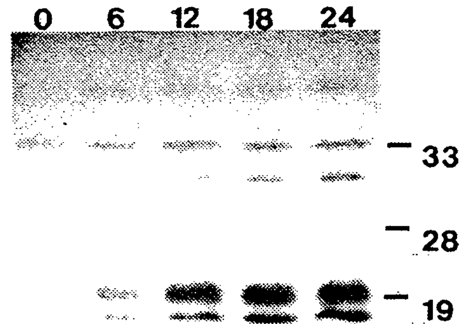


Figure 6: Western immunoblot analysis of HEM45 levels in HeLa cell monolayers which are in various stages of confluence.

The time at which cells were collected is indicated on the top of the panel (0, 6, 12, 18, and 24 hr). At zero hours, the monolayers were approx. 30% confluent. Lysates were prepared from HeLa cells ($2x10^5$ cells/lane), electrophoresed on 15% SDS-polyacrylamide gel, transferred to Immobilon P membrane, and challenged with rabbit anti-peptide made against HEM45 sequence 3-16 (1:500 dilution). The secondary antibody was donkey anti-rabbit IgG-alkaline phosphatase (1:2,000 dilution), and developed for ECL detection (Amersham). The secondary was donkey anti-rabbit IgG-alkaline phosphatase (1:2,000 dilution), and developed for ECL detection (Amersham). The relative migration positions of molecular weight markers are noted at the right of the panel (x 1,000 daltons).

IMMUNOHISTOCHEMISTRY FOR
HEM45 IN HeLa CL1 CELLS
(X826 ANTI-N-TERMINAL POLYCLONAL)

Figure 7: Preliminary analysis of cellular distribution of HEM45 in HeLa Cells.

Cells were plated to chamber slides (Lab-Tek) at low seeding density and allowed to attach. Cells were formaldehyde fixed and permeablised with triton-X-100. Cells were immunostained with the Vecstain (Burlingame CA) ABC system using X-826 rabbit polyclonal antiserum to the HEM45 3-16 peptide, and peroxidase based color development. CL1 is a puromycin resistant derivative of HeLa. It has not been transfected with HEM45 constructs.