A Novel Nuclear Antigen

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Abstract

The human oestrogen receptor (hER) mediates some effects of the steroid hormone oestrogen and functions as a ligand-dependent transcription factor in the nuclei of oestrogen-sensitive cells. The measurement of hER levels in breast cancer biopsies provides useful clinical information regarding therapy and prognosis. The current study describes a monoclonal antibody raised against hER aa 497-507 which recognises a novel nuclear antigen.

Monoclonal antibodies were raised by immunising mice with a synthetic fragment of the hER (aa 497-507) conjugated to keyhole lymphocyte haemocyanin. Thirty antibody-secreting hybridomas were identified. Hybridoma supernatants were characterised by enzyme-linked immunosorbent assay (ELISA), immunological staining using MCF-7 cells, binding studies, and SDS-PAGE Western blotting.

One supernatant (15F6) displayed nuclear staining in fixed MCF-7 cells. Staining could be abolished by pre-incubation of the supernatant with the aa 497-507 peptide and peptide conjugates, but not with an unrelated hER peptide (aa 256-275). This antibody also stained the nuclei of hER negative breast cell lines MDA-MB-231 and MDA-MB-330, the breast cell line T47D, and liver cell line HepG2. Immunological staining of human tissue sections reveal the antigen to be present in the nuclei of keratocytes in skin and tubule and luminal endothelial cells of the kidney. The antibody identified a 120 kD band on Western blots with cytosols prepared from human breast cell lines and in solubilised cells. The antibody does not precipitate 16α-iodooestradiol-labelled ER from MCF-7 cells.

Expression-linked screening of the MCF-7 cDNA library with antibody 15F6 identified nine positive clones. Antibody staining could be blocked by pre-incubating the antibody with hER aa 497-507-BSA conjugate, but not with an unrelated hER peptide conjugate. The (260 bp) clones were found to be identical. Submission of sequence to BLAST protein and nucleotide databases revealed a lack of homology to known proteins and genes. Sequence was matched to expressed sequence tags (ESTs) from brain, liver/spleen, uterus, ovary, colon, heart, and placenta.

To further define the epitope of antibody 15F6, the sequence was translated and three peptides containing potential epitopes, comparable to the hER aa 497-507 region, were synthesised and tested by ELISA. The putative epitope was shown to be contained within one of these peptides.

Introduction

1.1 The Human Oestrogen Receptor (hER)

The human oestrogen receptor (hER) is a member of the steroid receptor superfamily, a family of related nuclear proteins that includes the receptors for other classes of steroid hormones, thyroid hormone, peroxisome proliferator, vitamin D, retinoids, and the orphan receptors (Kuiper *et al.*, 1996; Evans, 1988). Collectively, these function as ligand-dependent transcription factors, playing a central role in mammalian development by regulating the expression of a variety of target genes (Beato, 1989).

The hER mediates some of the effects of oestrogen, a hormone which plays a role in many developmental and physiological processes, including embryonic and fetal development, development of female secondary sex characteristics, female reproductive cycle, maintenance of pregnancy, and fertility. The physiological responses generated by this hormone are tissue-specific and include tissue differentiation, growth, protein synthesis, and secretion. Knockout mice lacking functional oestrogen receptor are infertile and show abnormal phenotypical changes associated with the gonads, mammary glands, reproductive tracts, and skeletal tissues (Korach, 1994).

The molecular cloning of the hER (Greene *et al.*, 1986) has allowed for detailed structural and functional analysis, including the assignment of the various functions of the receptor (ligand binding, dimerisation, nuclear translocation, DNA binding and transactivation) to regions of the protein based on amino acid sequence homology to other members of the steroid receptor superfamily (Lewis *et al.*, 1995, Truss & Beato, 1993). The hER has been divided into six functional domains denoted A-F (Kumar *et al.*,1987)(Figure 1).

The hER resides primarily in the nuclei of oestrogen-responsive cells, both in the presence and absence of its ligand (King & Greene, 1984). In the absence of hormone, the receptor is complexed with a 90 kDa heat-shock protein (HSP90) and other proteins. Hormone binding induces a conformational change in the receptor which leads to release from HSP90, dimerisation, and binding to oestrogen-responsive elements (EREs) in the DNA of oestrogen-sensitive genes (Lewis *et al.*, 1995).

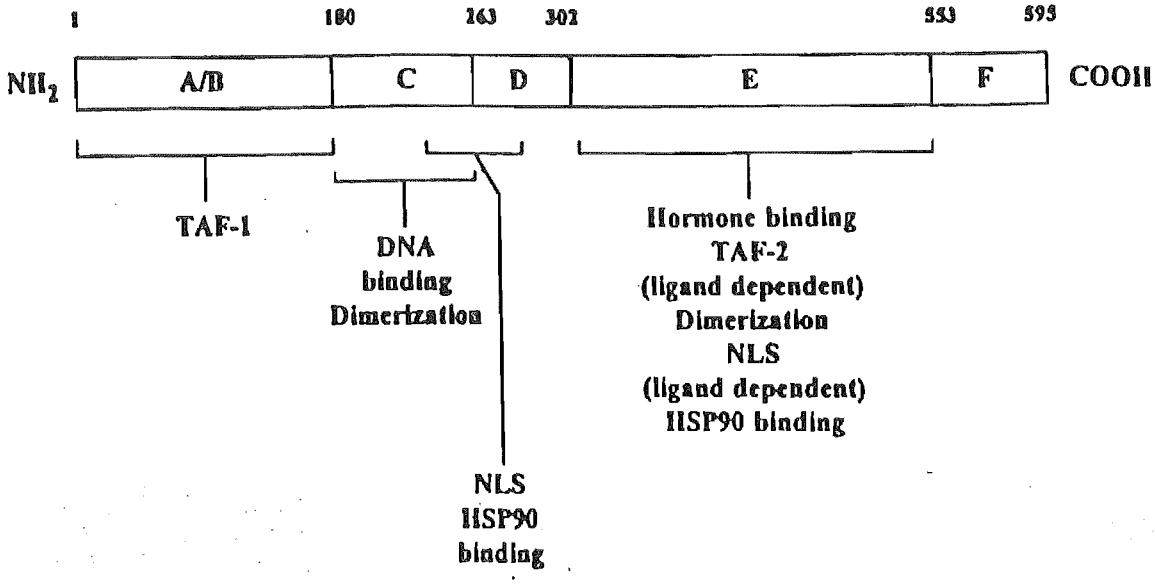

Figure 1. Structural and functional domains of the human oestrogen receptor (hER). TAF; transcription activation function, NLS; nuclear localisation signal (Modified from Levenson & Jordan, 1994).

1.2 Clinical Utility of the Human Oestrogen Receptor

Epidemiological studies have revealed an association between lifetime exposure to oestrogen and incidence of breast cancer (Vessey, 1989; Henderson, 1988). There is a strong body of evidence pointing to oestrogen as being involved in the promotion of breast cancer and tumour growth (Lemieux & Fuqua, 1996). Endocrine therapy is designed to counteract this process and consists of the administration of anti-oestrogens which bind the hER without causing transcriptional activation.

Clinically, the most important application for the measurement of hER content is to identify patients with breast cancer who may benefit from endocrine therapy. In addition, hER status may provide prognostic information and add to our understanding of hER biology. With regards to prognosis, the presence of hER in tumours has been associated with prolonged disease-free and overall survival (Knight *et al.*, 1977).

Generally, tumours which lack ER and progesterone receptor (PR) behave more aggressively than ER/PR positive tumours (Sluyser, 1992). The predictive value of ER status with regard to response to endocrine therapy and prognosis is not completely reliable; measurement of PR status further improves the ability to predict clinical outcome. For instance, approximately 75% of patients with advanced hER/PR positive tumours respond to the anti-oestrogen drug Tamoxifen, whereas 10% of patients with hER/PR negative tumours respond to the same extent (Lemieux & Fuqua, 1996; Osborne *et al.*, 1980). The remaining hER positive tumours are thought to fail to respond to endocrine therapy due to the emergence of a sub-population of mutated and truncated oestrogen receptors which function constitutively, ie enhance transcriptional activity independent of the hormone. In this respect, constitutively active receptors resemble the oncogenes in their activity (Sluyser & Mester, 1985).

1.3 Measurement of the Human Oestrogen Receptor

The advent of hybridoma technology has led to the development of monoclonal antibodies. These are extremely powerful research tools due to their high specificity of binding, their homogeneity, and their ability to be produced in unlimited quantities. The basis of hybridoma technology consists of the fusion of antibody-secreting cells isolated from the immunised animal with myeloma cells to provide an immortal somatic cell hybrid that secretes antibodies (Kohler & Milstein, 1975)(Figure 2). Antibodies made by this process are now utilised in various immunochemical techniques for the detection, purification, quantification and investigation of the hER, and provide several advantages over the previously used ligand binding approach, in which a radio-labelled hormone serves as a marker for the receptor to which it binds.

The limitations associated with ligand binding techniques include the nature of the interaction between ligand and receptor (the interaction is non-covalent, increasing the likelihood of ligand displacement during experimental manipulations), failure of the ligand to detect receptors already occupied by endogenous hormone, and failure of the ligand to bind to partially denatured, truncated or mutated receptors that have lost their hormone binding ability (Greene & Jensen, 1982). Furthermore, ligand binding methods such as the dextran-coated-charcoal (DDC) assay require large specimens, and results from the assay

are dependent on cell viability and cell types present (ratio of stromal to malignant cells)(Al Saati *et al.*, 1993).

These limitations are largely overcome by the use of antibody-mediated methods. An additional advantage conferred by using monoclonal antibodies is the ability to obtain information about receptor content of individual cancer cells from biopsy samples. This is desirable in light of the cellular heterogeneity in both hER content and structure associated with breast tumour evolution.

Commercial monoclonal antibodies to the hER are available (Al Saati *et al.*, 1993), however, these are costly. As part of an ongoing monoclonal antibody-generation program at the Steroid Laboratory, Clinical Biochemistry Unit, Canterbury Health Laboratories, Christchurch, New Zealand, an attempt was made to develop a monoclonal antibody to the hER suitable for routine immunochemical quantification of hER content in tumour biopsies. Such an antibody has the potential to enhance/replace the ligand-binding assay that is currently in use at the Laboratory.

To date, both monoclonal and polyclonal antibodies to the hER have been generated using purified hER (King & Greene, 1984), hER peptide-carrier protein conjugates (Traish *et al.*, 1990, Poutanen *et al.*, 1992), or recombinant hER as the immunising antigen (Al Saati *et al.*, 1993). While all three strategies were attempted within the Steroid Laboratory, only the peptide-conjugate approach proved successful.

1.4 The Production of Anti-peptide Antibodies

The use of anti-peptide antibodies (raised against synthetic peptides) may be preferable over antibodies raised against purified antigens. Synthetic peptides can be used to raise antibodies directed against previously uncharacterised proteins, given that some of the sequence for the protein of interest is available. If more knowledge is available about the protein in question, particular regions of a protein can be targeted specifically for antibody production. Such site-specific antibodies have both clinical and research applications (Lerner, 1982).

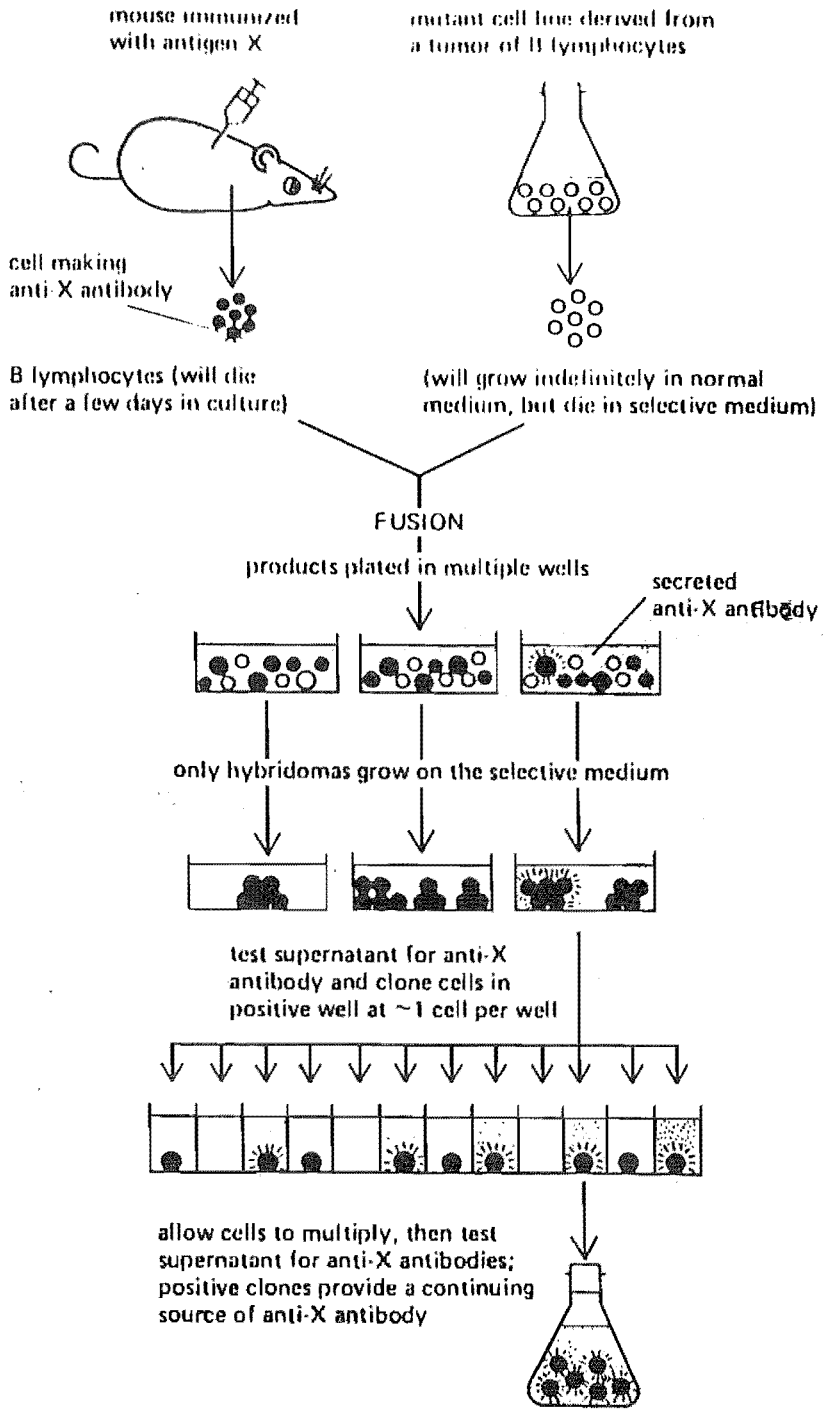

Figure 2. The generation of monoclonal antibodies. Mice are immunised intraperitonally with peptide-carrier protein conjugates at 4 week intervals. Ten days following a third injection, the spleen is aseptically excised and splenic lymphocytes fused with mouse myeloma cells to produce immortal hybridomas. Successful fusions (hybridomas) are selected for through use of a selective media, such as AAT (adenine, aminopterin, thymidine) which blocks nucleotide synthesis. Genetic information imparted by myeloma cells allows hybridomas to activate an alternate synthetic pathway, and hence multiply. Hybridomas of interest can be isolated and cloned by limiting dilution techniques and screened by ELISA, radioimmunoassay, immunofluorescence or immunocytochemistry (Modified from Alberts *et al.*, 1989).

Previous knowledge of protein structure can overcome the main limitation of anti-peptide antibodies; while they may bind the denatured protein extremely well, they often fail to recognise the protein in its native configuration. The coupling of the peptide to a carrier protein overcomes a second problem which can be encountered with anti-peptide antibodies, namely that the peptide itself may not elicit an immune response, due to the small size.

The peptide sequence should be carefully selected in order to obtain an antibody which recognises the native protein. Hydrophilicity, "mobility" and position of the sequence within the native protein are all useful criteria. Hydrophilicity is required but not sufficient to predict whether a given sequence will be located on the surface of a protein (Hopp & Woods, 1981). The "mobility" of the amino acid residues refers to their relative temperature as determined by NMR and X-ray crystallography. It has been suggested that high temperature regions represent areas of increased flexibility which are more likely to be epitopes (Westhof *et al.*, 1984). The position of the sequence is also informative; sequences present at either the amino- or carboxy-terminus are often exposed, making good targets (Harlow & Lane, 1988). Regions which are known to contain binding sites are also an obvious choice.

The hER aa 497-507 region was chosen as a likely epitope due to its hydrophilic nature, proximity to a putative dimerization domain (Lewis *et al.*, 1995) and unique sequence as determined by homology searches. The latter characteristic ideally leads to the development of an antibody which does not cross-react with other proteins.

1.5 Antibody Characterisation

For anti-peptide antibodies, it is necessary to investigate each antibody's ability to recognise the native protein in addition to the immunizing peptide. The enzyme-linked immunosorbent assay (ELISA) enables the recognition between antibody and peptide to be examined and quantified. The indirect ELISA is designed to quantify the amount of antibody that is able to bind specifically to antigen immobilized on a solid phase (microtitre plate) through use of a labelled second antibody and substrate (Figure 3). Immunoassays such as the ELISA, are amongst the most sensitive analytical techniques available for the clinical detection of antigens and antibodies in body fluids (Kricka, 1994).

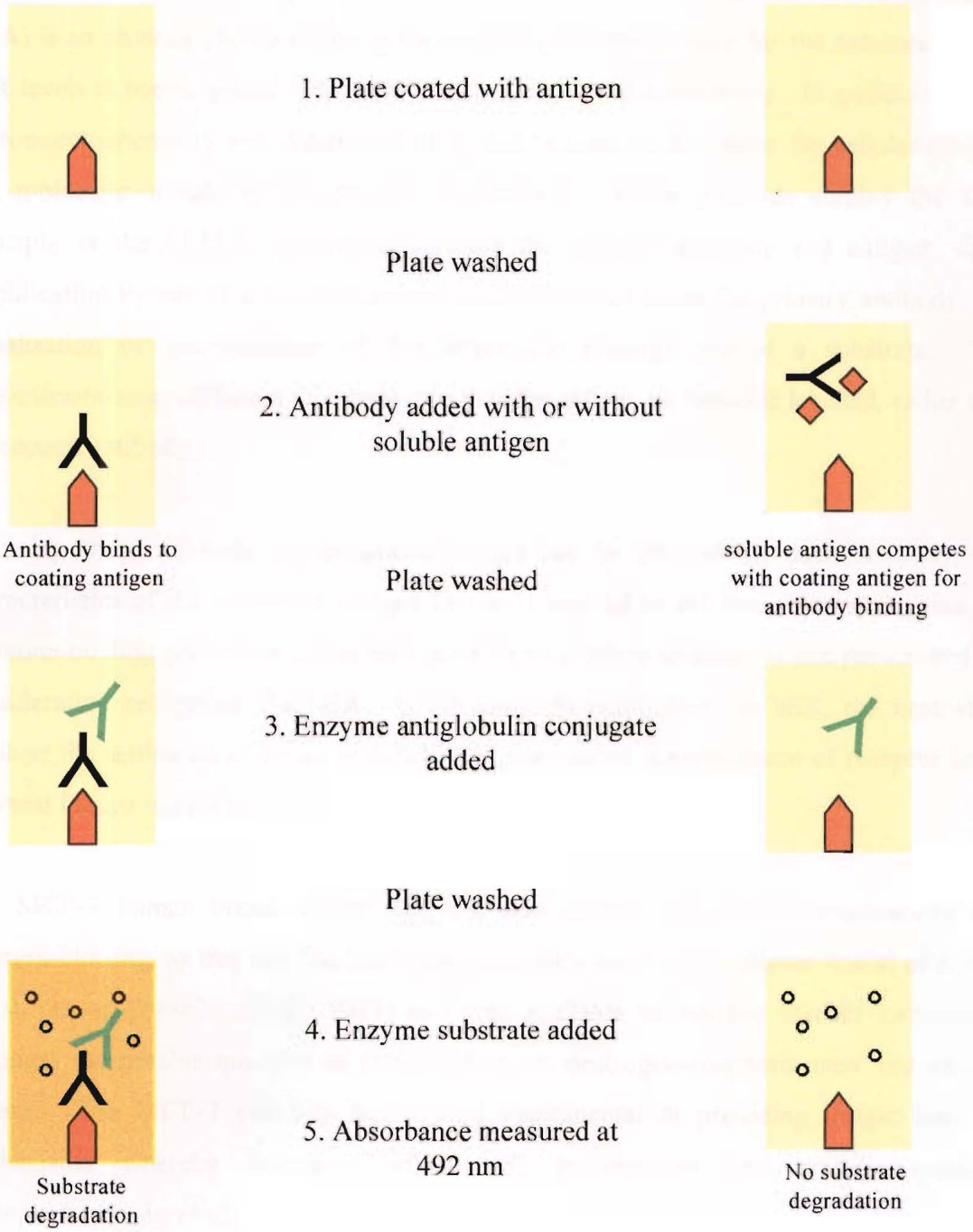

Figure 3. The indirect enzyme-linked immunosorbent assay (ELISA). The assay allows monoclonal antibodies of interest to be tested for their ability to recognise the antigen they were raised against (eg hER aa 497-507). In a variation of the assay (right-hand side), competitive dose-response curves can be generated by adding increasing amounts of soluble antigen with the monoclonal antibody.

To determine whether recognition of the native hER is occurring, the radioimmunoassay (RIA) is an obvious choice as this is the method currently utilized for the measurement of hER levels in breast cancer biopsy samples at the Steroid Laboratory. In addition to this, immunocytochemistry and Western blotting can be used to determine the cellular location and molecular weight of the antigen respectively. These methods employ the same principle as the ELISA; interaction between the primary antibody and antigen, signal amplification by use of a labelled second antibody which binds the primary antibody, and visualisation or quantification of this interaction through use of a substrate. The radioimmunoassay differs in this respect in that the antigen or ligand is labelled, rather than the second antibody.

By using these methods, an integrated profile can be derived for each antibody, and characteristics of the respective antigen can be compared to the known size, location and hormone-binding properties of the hER in order to confirm whether or not the antibody in consideration recognises the hER. If the antibody recognises the hER, the next stage involves the setting up of an assay suitable for the routine determination of receptor levels in breast biopsy samples.

The MCF-7 human breast cancer cell line was utilised for immunocytochemisty and Western blotting, as this cell line has been extensively used as a research model of human breast cancer (Brooks *et al.*, 1984) and was available within the Steroid Laboratory. Amongst the notable qualities of this cell line are oestrogen-responsiveness and ease of culture. The MCF-7 cell line has proved instrumental in providing insight into the mechanisms whereby hormones affect cell proliferation and protein synthesis (Katzenellenbogen *et al.*, 1987).

1.6 Aims of the Study

The initial aim of the study, as envisaged by the Steroid Laboratory, was to obtain a monoclonal antibody suitable for the routine determination of hER levels in breast tumour biopsy samples, using hER aa 497-507 as the immunizing antigen. While no such antibody was found, during the early stage of the antibody characterisation process it became apparent that one of the antibodies (15F6) recognises an antigen other than the hER, providing an opportunity for further research.

Homology searches of hER aa 497-507 in both peptide (BLASTX) and nucleotide (BLASTN) databases revealed homology to oestrogen receptors from chicken, mouse and rat, wheat gamma-gliadin precursor and yeast negative growth regulatory protein NG GR1 (Figure 4). This lack of homology to known human proteins was taken as an indication that this protein could be novel.

Specifically, the experiments were designed to:

- characterise a panel of monoclonal antibodies for their ability to recognise the hER.
- verify that antibody 15F6 does not recognise the hER by immunological techniques.
- examine the cellular location of the antigen recognised by antibody 15F6 in available human cell lines and tissues by immunological staining.
- derive the epitope sequence recognised by monoclonal antibody 15F6 by ELISA competition experiments.
- screen a human breast cancer cDNA library with antibody 15F6 to obtain sequence for the respective antigen.

The data presented in this thesis support the hypothesis that antibody 15F6 recognises a novel nuclear antigen which is present in a wide variety of human tissues.

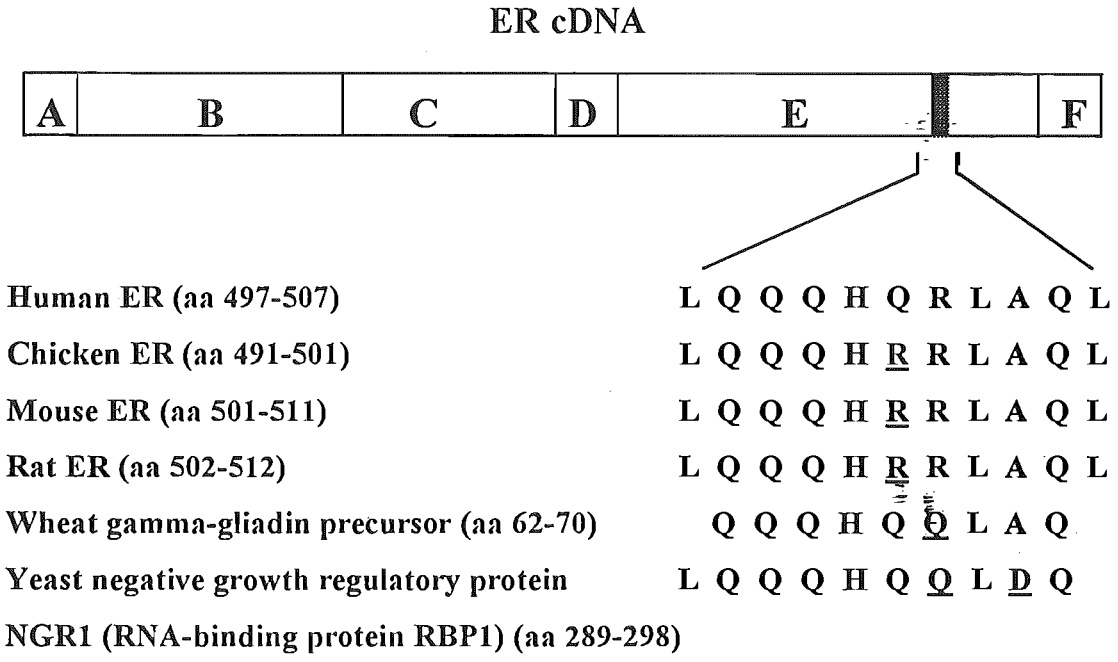

Figure 4. Location and interspecies sequence comparison of hER aa 497-507 synthetic peptide. The chosen peptide corresponds to a 11-aa sequence lying in region E of the hER. The peptide sequence (using single letter aa abbreviations) is compared to equivalent ER sequences of the chicken, mouse and rat. Two non-human proteins with homology to this peptide are also shown. Non-identical amino acids are underlined. Sequence was obtained from a BLASTP search.

2.1 Reagents and Materials

All reagents used were of analytic (AR) grade; supplier details are listed in Appendix A. The sources of materials used are indicated in the text, with full supplier details listed in Appendix A.

2.2 Buffers and Media

Composition details of buffer, media and other solutions can be found in Appendix B.

2.3 Peptides

The hER aa 497-507 sequence was synthesized by Multiple Peptide Systems, and was greater than 96% pure by reverse phase HPLC. The peptides used for epitope definition studies were obtained from Chiron Mimitopes Pty. Ltd, and were approximately 50% pure as determined by mass spectrometry (Figure 5).

hER aa 497-507	L Q Q Q H Q R L A Q L - NH_2
Peptide # 1	G Q Q Q S Q H G G S H P - NH_2
Peptide # 2	V Q H H Q H Q A A Q A L H L A S P Q Q Q S A I Y H - NH_2
Peptide # 3	T Q S P Q N S F P A A Q Q T V F T I H P S H V Q P - NH_2

Figure 5. Peptide sequences used for antibody competition experiments and ELISA epitope definition studies.

2.4 Conjugation of Peptides

Peptides were coupled to keyhole lymphocyte haemocyanin (KLH), bovine serum albumin (BSA), or thyroglobulin (TG) with glutaraldehyde according to the method of Harlow and

Lane (1988). Glutaraldehyde is a bifunctional coupling reagent that links two compounds primarily through their amino groups, forming a relatively stable linkage.

Briefly, solutions of peptide (5 mg/ml in PBS) and carrier protein (3.5 mg/ml in PBS) were prepared, and equal volumes (1 ml of each solution) were added to a 5 ml vial containing a stirring bar. To this solution, an equal volume (2 ml) of 0.2% glutaraldehyde (in PBS) was added drop-wise, and allowed to incubate for 1 h at room temperature with constant stirring. This was followed by the addition of 1 ml of 1 M glycine (in PBS) and a further incubation of 1 h. PBS was filter-sterilised prior to use. Following coupling, the conjugates were dialysed overnight at 4°C against 1 L PBS and stored in aliquots at -20°C until required for immunisation, screening of culture supernatants or other studies.

Coupling of the hER aa 497-507 peptide to KLH (for immunisation) and BSA (for screening of supernatants using ELISA) was performed by Dr John Lewis, Steroid Laboratory.

The coupling procedure described above was also utilised for making the peptide conjugates used in ELISA epitope definition studies. In this case, peptides were coupled to TG.

2.5 Immunisation and Cell Fusion

Male RBF/DN mice (obtained from the Christchurch School of Medicine Breeding Colony, Christchurch, New Zealand) were immunised intra-peritoneally with 50 µg of aa 497-507-KLH conjugate in Freund's complete adjuvant (Sigma) at four week intervals. Ten to twelve days following a third or fourth injection, the spleen was aseptically excised and the splenic lymphocytes fused with FOXNY mouse myeloma cells using 50% PEG at a ratio of 5:1 as previously described (Lewis *et al.*, 1989). When hybridoma growth could be detected, supernatants were tested for antibody-binding activity using ELISA. Hybridomas producing potentially interesting antibodies were cloned by limiting dilution and characterised. Immunisation, cell fusion and cloning were performed by Dr John Lewis, Steroid Laboratory.

2.6 Enzyme-linked Immunosorbent Assay (ELISA)

Supernatants were screened by ELISA using 96-well microtitre plates (Nunc.) coated with the hER aa 497-507-BSA conjugate. To coat each plate, 10µl of peptide-BSA conjugate was added to 10ml of 6 M aqueous guanidine hydrochloride and 100µl added to each well. Following overnight incubation at 4°C, the plates were washed (4x) with wash buffer, and "blocked" using phosphate buffered saline (PBS) assay buffer, 150µl/well. Following blocking, 1 h at 20°C, the solution was removed and replaced with fresh PBS assay buffer, 50µl/well, followed by 50µl of culture supernatant/well. Plates were incubated for 2 h at room temperature and then washed (4x). The second antibody, goat antimouse Ig peroxidase (Amersham), was added (100µl/well), at a dilution of 1:2000 in PBS assay buffer for a further incubation of 2 h at room temperature. Substrate solution (100 µl/well) was added after final washing of the plates. Colour development was terminated by the addition of 100µl/well of 1.25 M H_2SO_4/well, and absorbance read at 492 nm.

Dose-response curves were generated on coated microtitre plates (as described above), with varying doses of soluble peptide conjugates (in PBS assay buffer) and supernatants from the appropriate clone (in PBS assay buffer), with subsequent processing as described. Supernatants giving good dose-response curves (DRCs) were diluted (1:10, 1:20, 1:40 and 1:80, in PBS assay buffer), and the experiment repeated in order to determine the most sensitive part of the curve.

2.7 Cell Lines and Tissue Culture

The MCF-7 cell line was obtained from Dr Ian Holdaway, Department of Endocrinology, Auckland Medical School, Auckland, New Zealand, and the oestrogen-receptor negative cell lines MDA-MB 231 and MDA-MB 330 from Dr Rob Sutherland, Garvan Institute, St Vincent's Hospital, Darlinghust, Australia.

Cells were grown in 50 ml culture flasks or on 8-chamber tissue culture microscope slides (Nunc.) in a humidified atmosphere containing 5% CO_2 in air at 37°C, and maintained in adherent monolayer culture in phenol red-free RPM1640 (Gibco) supplemented with 10% fetal calf serum and 2 mM glutamine. The culture media was replaced with fresh media every 3 to 4 days and cells were grown until nearly confluent. Prior to harvesting the cells, media was removed and the cells were gently rinsed with PBS (3x). Cells were harvested

by vigorous flushing with a pasteur pipette and cold PBS. Cells were pelleted by centrifugation at 2000 RPM for 20 min, the PBS aspirated, and cell pellets stored at -20°C until use for SDS-PAGE Western blotting.

Cytosols for SDS-PAGE Western blotting were prepared by resuspending cell pellets in 500µl Tris-EDTA buffer and homogenising the cell suspension manually in a 10ml Teflon homogeniser on ice. The homogenised solution was decanted into microcentrifuge tubes, spun at 14,000 RPM for 10 min at 4°C, and the supernatants transferred to fresh microcentrifuge tubes and stored at -20°C.

2.8 Immunological Staining

Hybridomas identified as positive by ELISA screening were further investigated by immunochemical and immunofluorescent staining. Breast cell lines were grown on 8-chamber tissue culture microscope slides until almost confluent. Prior to fixation, the medium was aspirated and adherent cells were gently washed with PBS (3x).

For immunofluorescence labelling, cells were fixed with 2% paraformaldehyde in PBS (300µl/well) for 30 min at 20°C. Following fixation, the cells were re-washed with PBS (3x) and incubated with fetal calf serum (FCS, 300µl/well) for a further 30 min to minimize non-specific binding of the antibody. Following aspiration of FCS, hybridoma supernatants were added (200µl/well) and incubated overnight at 20°C. The cells were gently washed with PBS (3x), and 300µl goat antimouse IgG/M fluorescein was added (1:100 in PBS containing 1% BSA, Tago) for a 3 h incubation at 20°C. After final washing with PBS (3x), the chamber lining and sealing gasket were removed and coverslips applied to allow for viewing under UV and white light using an oil immersion lens. All washing and antibody solutions added to the cells were passed through sterile 0.45µm Millipore filters (Millipore Corporation).

Breast cells processed for immunoperoxidase staining were fixed with 100% ethanol (300 µl/well) for 10 min at 20°C, followed by methanol (containing 0.03% H_2O_2, 300 µl/well) for 30 min at 4°C. The cells were gently washed with PBS (3x), and incubated with NSS (normal sheep serum, 1:10 in PBS, 300 µl/well) for 20 min at 20°C. Following aspiration of serum and washing with PBS (3x), hybridoma supernatants were added (1:10 in PBS,

200 µl/well) for a 3 h incubation at 20°C. Cells were washed with PBS (3x), and sheep antimouse biotin was added (300 µl/well, 1:200 in PBS containing 1% NSS, Amersham). Following incubation for 30 min at 20°C, cells were washed with PBS (3x) and avidin-biotinylated HRP complex was added (300 µl/well, 1:200 in PBS containing 1% NSS, Amersham) for a 30 min incubation at 20°C. After final washing with PBS (3x), diaminobenzidine (DAB) substrate solution was added. Colour development was terminated by immersing the slides in distilled H_20 after the desired staining intensity was achieved (10-20 min). The chamber lining and sealing gasket were removed and coverslips were applied to allow for viewing under the light microscope. The location (nuclear, cytoplasmic, both or neither) and intensity of staining were noted for each antibody.

Of interest were antibodies displaying predominantly nuclear staining (since the hER is localised exclusively in the nuclei of MCF-7 cells). Such antibodies were used for further immunoperoxidase staining experiments on two human breast cancer cell lines which do not express the hER (MDA-MB-231 and MDA-MB-330). Staining was compared to that of hER positive MCF-7 cells.

Antibody 15F6 was also used to stain two available adherent human cell lines; the human breast cell line T47D and the human liver cell line Hep G2, obtained from the Department of Cell Biology, University of Auckland, Auckland, New Zealand and the Wistar Institute, Philadelphia, USA, respectively.

Immunohistological staining with antibody 15F6 was performed on mounted human skin and kidney sections kindly provided by the Haematology/Immunology Research Group, Christchurch School of Medicine, Christchurch, New Zealand. Following fixation (10 min in ice cold acetone), sections were dried (30 min under moving air) and blocked using 10% human serum in PBS (5 min). The blocking solution was replaced with antibody 15F6 (1:10 in TBS) and subjected to a 30 min incubation period, after which slides were washed with TBS (3x). Biotinylated goat antimouse Ig (1:200 in TBS) was subsequently applied for a further 30 min period, followed by washing with TBS (3x) and the addition of streptavidin-alkaline phosphatase (1:200 in TBS, Dako). After a final washing step, slides were immersed in Fast Blue substrate solution. Colour development was monitored under a light microscope, and the reaction stopped by washing with PBS.

Skin sections were double-labelled with 15F6 and VM-2, the latter of which allows for definition of the keratinocyte layer through staining of basal keratinocytes. VM-2 staining was performed as descibed above, while 15F6 staining was performed with goat antimouse Ig peroxidase (1:200 in TBS, Dako) and AEC subtrate. Slides were photographed on Kodak Gold 100 ASA film using an Olympus BX-50 equipped with a PM-30 camera system.

2.9 Binding Studies

Oestrogen receptor positive cytosols labelled with 16α-iodo-oestradiol (>1500 Ci/mmol, Amersham) were used to investigate hybridoma supernatants deemed positive by ELISA. Human breast tissue cytosols were the 36000 *g* supernatants after homogenisation of tumour tissue in Tris-EDTA buffer. Protein content of cytosols was approximated by a modified Bradford assay (Bearden, 1978).

Briefly, cytosol was diluted to 1 mg/ml protein with Tris-EDTA buffer and an equal volume of 16α-iodo-oestrodial diluted in Tris-EDTA buffer (200,000 dpm/ml) was added for an overnight incubation at 4°C. Aliquots of labeled cytosol (200µl) were then incubated with 100µl of supernatant (1:10 in Tris-EDTA buffer) for 3 h at 4°C followed by 100µl donkey antimouse/rat IgG cellulose (Sac-Cel, IDS) for 30 min at 4°C. One ml of Tris-EDTA buffer was then added and the tubes centrifuged, supernatant aspirated and the pellet counted. Binding studies using 15F6 antibody and 16α-iodo-oestradiol were also carried out at pH 6.5 and also in the presence of 0.4 M KCl. These conditions have been shown to minimise the formation of hER dimers (Thole *et al.*, 1991), an occurrence which could potentially result in masking of the antibody binding site and hence prevent antigen-antibody interaction.

Similar experiments were also carried out with 16α-iodo-oestradiol labeled cytosols which were treated with dextran-coated charcoal (DCC) prior to the addition of hybridoma supernatants. This has the effect of removing any unbound oestradiol. Controls were always run consisting of oestrogen receptor positive and negative cytosols and separation with dextran-coated charcoal rather than hybridoma supernatant.

2.10 Western Blot Analysis

Proteins from solubilised whole cells or cytosolic extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide gels (Laemmli, 1970), and electrophoretically transferred (2 h at 70 V) to nitrocellulose (Schleicher & Schuell) using a Bio-Rad Mini Protean II system. Lanes containing molecular weight markers were cut off and stored in the dark at 4°. The filters for immunostaining were blocked overnight in Tris-buffered saline (TBS) containing 5% skim milk powder.

The nitrocellulose was cut into strips and incubated with the individual hybridoma supernatants (1:10 in TBS) for 3 h. Strips were washed with TBS (3 x 5 min), and incubated with sheep antimouse IgG peroxidase (1:10,000 in TBS, Amersham) for 1 h. All incubation and washing steps were at room temperature with gentle agitation. Following washing (3 x 5 min with TBS), the nitrocellulose strips were taken into a darkroom, immersed in enhanced chemiluminescence (ECL) substrate for approximately 5 seconds, blotted on 1M Whatman paper to remove excess substrate and exposed for 1 min on Kodak X-OMAT AR film (Eastman Kodak Co.). After this short exposure, the filters were re-exposed for a further 20 min and developed at the Radiology Services, Christchurch Public Hospital, Christchurch, New Zealand. Protein bands were sized by comparison to molecular weight markers (Kaleidoscope prestained standards, BioRad).

2.11 Isotyping of Antibody 15F6

The antibody was isotyped according to instructions provided with the Mouse Monoclonal Antibody Isotyping Kit (Amersham). The kit allows for accurate determination of class (IgA, IgG or IgM), the IgG subclass (G1, G2a, G2b or G3) and light chain type (κ or λ) of the antibody through the use of nitrocellulose typing sticks which contain goat antibodies specific for the different types of peptide chain.

Briefly, a typing stick was incubated with the hybridoma supernatant solution (1:10 in TBS) in a capped tube and incubated for 15 min at room temperature with agitation. After removal of the supernatant solution and washing (2 x 5 min with TBS), sheep antimouse peroxidase (1:500 in TBS) was added and allowed to incubate at room temperature with agitation for 15 min. Following removal of the antimouse peroxidase solution, the typing stick was washed (2 x 5 min with TBS) and substrate was added for a further 15 min

incubation period. The substrate used was the ELISA substrate (refer Appendix) instead of the substrate included with the kit (4-chloro-1-napthol). After removal of the substrate, the typing stick was rinsed with distilled water (3 x) and the result (indicated by the appearance of purple coloured lines on the appropriate sections of the stick) interpreted immediately.

2.12 Affinity Chromatography

Antibody 15F6 was purified using an affinity column consisting of rat antimouse IgM coupled-Sepharose 4B (Pharmacia). Following overnight coupling, the antibody was eluted from the column with 2 M KI and dialysed overnight against PBS. The purified antibody was subsequently coupled to CNBr-activated Sepharose 4B (Pharmacia),(Lewis, 1983). Prior to use, the coupled gel was washed with TED buffer.

The gel was added to cytosols prepared from MCF-7 cells (in TED buffer) and incubated overnight at 4°C with gentle shaking. This mixture was transferred to a minicolumn and rinsed alternately with TED and TED containing 0.5 M NaCl to remove protein bound non-specifically to the column. Acetic acid (pH 2.5) was used to elute the "purified' protein fraction, which was dialysed overnight against distilled water. This solution was subsequently freeze-dried.

2.13 ELISA and Western Blotting of the Purified Protein Extract

A simple ELISA of the protein extract purified by affinity chromatography was performed on a microtitre plate was coated with hER aa 497-507-BSA conjugate. The protein extract was diluted serially in PBS assay buffer (1:2, 1:4, 1:8, 1:16 and 1:32) and 50µl of each of the dilutions added/well, followed by the addition of 50µl of antibody 15F6 (diluted 1:10). PBS assay buffer was substituted for protein extract in case of the control. The assay was processed as prescribed in section 2.6.

A small amount of the extract was diluted in electrophoresis buffer and subjected to SDS-PAGE. Following SDS-PAGE, half of the gel was stained for protein with Coomassie Blue while the remaining half was used for Western blotting. SDS-PAGE and Western blotting were performed in accordance with section 2.10.

2.14 Immunodiffusion and Immunoelectrophoresis of the Purified Protein Extract

Immunodiffusion and immunoelectrophoresis were performed according to the methods of Turner and Hulme (1971). These techniques rely on the establishment of concentration gradients of antigen and antibody in a transport medium, such as an agarose gel, where immunoprecipitation can be observed at the antigen/antibody interface. Both methods are excellent tools for investigating antigens.

For immunodiffusion, 1% agarose gels were made by adding 500 mg of agarose in 50 ml of Tris-barbitone buffer and boiling this for 2 min in order to completely dissolve the agarose. After cooling to approximately 50°C, the gel was poured onto an 8 cm x 10 cm glass plate to a thickness of 2-3 mm. A period of at least 1 h was allowed for polymerisation of the gel, after which a pattern of small holes (3-4 mm in diameter) were punched in the gel; a central well surrounded by up to three peripheral wells, each separated from the central well by a distance of 3-4 mm. The protein extract (dissolved in Tris-barbitone buffer) was added to the central well, and antibody solutions of interest (anti-IgM, anti-human serum, anti-ceruloplasmin and antibody 15F6) to surrounding wells. Diffusion was allowed to proceed overnight under high humidity conditions, after which the gel was pressed overnight under several layers of filter paper, a stack of paper towels and a 500 g weight. The gel was subsequently fixed (10-15 min at 70°C), stained with Coomassie Blue (10 min), rinsed with distilled water, destained (30 min with agitation), and dried (2-3 min at 70°C).

For immunoelectrophoresis, a 1% agarose gel was prepared (as described above), in which two small holes were made, approximately 2 mm accross, 10 mm apart and 3 cm from the edge of the gel (longitudinal orientation of gel). Human serum (containing bromophenol blue) was added to one of the wells and the protein extract to the other. The gel was placed in a gel tank and connected to the barbitone buffer reservoirs by paper wicks (3M paper, several layers). Electrophoresis, which distributes proteins in bands according to their respective mobilities, was performed for 2 h at 100 V.

Following electrophoresis, a 2 mm wide longitudinal trough was cut at an equal distance between the two wells and filled with anti-human serum. Diffusion was allowed to proceed overnight under high humidity conditions, after which the gel was pressed, stained, destained and dried (as described above). Diffusion of the serum and antiserum results in

the formation of a series of characteristic precipitin arcs; the position of these arcs (ie mobility of respective proteins) can be compared to any arcs generated as a result of immunoreactivity between the protein extract and anti-human serum, hereby providing a means whereby contaminant human serum proteins can be detected.

2.15 Bacterial Host Strains

Bacterial strains used in this study were obtained from glycerol stocks kept at -80°C by Dr Martin Kennedy, Christchurch School of Medicine, Christchurch, New Zealand (Table 1). Bacteria were streaked onto agar plates and kept at 4°C for up to one month, at which point a single colony was taken and streaked onto a fresh plate. Single colonies were taken from this stock plate for overnight liquid cultures as required. Strains were cultured under conditions described in the appropriate sub-sections.

E.coli Y1088 was used for DNA preparation and *E.coli* Y1090 for expression-linked library screening. This strain has a mutation in the *lon* protease to allow for increased fusion protein stability. *E.coli* DH5α was used for cloning.

Strain	Ref	Genotype
DH5α	Woodcock, *et al.*(1989)	F' *endA1 hsdR17* (r_k - m_k+) *supE44 thi-1 RecA1 gyrA* (Nalr) *relA1 Δ(laclZYA-argF)U169 deoR* (ϕ80*dlacΔ(lacZ)M15)*
Y1088	Huynh, *et al.* (1985)	F- *Δ(lac)U169 SupE SupF hsdR(r_k - m_k+) metB trpR fhuA21 proC::Tn5* (pMC9; Tet^r Amp^r) NOTE: pMC9 is pBR322 with $lacI^q$ inserted.
Y1090	Huynh, *et al.* (1985)	F- *Δ(lac)U169 lon-100 araD139 rpsL(*Str^r*) supR mcrA trpC22::Tn10* (pMC9; Tet^r Amp^r)

Table 1. Genotypes of *Escherichia coli* strains

2.16 Antibody Screening of the MCF-7 cDNA Expression Library

Antibody probes serve a dual function; they allow for the cloning of genes as well as the isolation of their respective protein products (Young & Davies, 1983). The library employed in this study was constructed in the expression vector λgt11. This vector permits insertion of foreign DNA into the β-galactosidase structural gene *lacZ* and promotes synthesis of hybrid proteins, which accumulate in *E.coli* strains defective in protein degradation (*lon* mutants). These hybrid proteins, when embedded on nitrocellulose membranes, can be screened with antibodies with relative ease.

Amongst the specific advantages of the λgt11 vector are its stability within the host cell and efficiency of response to induction (consisting of a rapid increase in copy number and high-level transcription of the foreign DNA. λgt11 (*lac5 nin5 c*1857 *S*100) was derived from λgt7 (*lac5 b522 nin5*) and λgt4 (*c*1857 *S*100 *nin5*) by Young and Davis (1983).

The MCF-7 cDNA library was obtained from Professor Pierre Chambon, Institiut de Chimie Biologique, Faculte de Medecine, 11 Rue Humann, 67085 Strasbourg Cedex, France.

The titre of the MCF-7 cDNA library, expressed as the number of plaque forming units (FPU) per ml, was determined by spotting 10μl drops of serially-diluted phage (in SM buffer) onto a bacterial lawn on an LB + amp plate, and counting the number of plaques visible after an incubation period of 6-8 h at 37°C.

The library was plated out on five 17 cm x 22 cm LB plates (containing 1 mM $MgCl_2$ and 50μg/ml Ampicillin). For each plate, 5 x 10^5 plaque forming units (PFU) were mixed with 1 ml of log phase Y1090 cells, pre-incubated for 20 minutes at 37°C and added to a 0.7% agarose LB overlay (containing 10 mM $MgSO_4$ and 50μg/ml Ampicillin). Plates were incubated for 6-8 h at 37°C and overlaid with nitrocellulose. The nitrocellulose membranes were immersed in a 10 mM solution of isopropylthio-β-D-galactosidase (IPTG) in distilled water and allowed to air dry prior to use. IPTG functions to induce protein expression by inactivating the bacterial *lac* repressor. After a further 3 h incubation at 37°C, the nitrocellulose membranes were blocked overnight in TBS (containing 5% skim milk powder).

For antibody probing, the membranes were treated as follows. After three 5 minute washes in TBS (to remove residual skim milk powder), the membranes were incubated with the 15F6 antibody (1:10 in TBS) for 3 h with gentle shaking at room temperature. Following washing (3 x 5 min), the second antibody was applied (goat antimouse IgG alkaline phosphatase, diluted 1:1000 in TBS, Tago) for a 1 h incubation. After a final wash (3 x 5 min), substrate (BCIP/NBT) was added. Colour development was stopped after reaching the desired intensity (<10min) by rinsing the membranes with distilled water. Membranes and library plates were stored at 4°C.

Immunoreactive clones were plaque-purified by eluting appropriately located agar plugs in SM buffer overnight at 4°C, replating the eluates at approx. 100-200 FPU/plate, and repeating the above procedure until all plaques were 'positive'.

To eliminate any false positives due to bacterial alkaline phosphatase production, the plaque-purified clones were re-screened with the more specific peroxidase substrate and second antibody sheep antimouse IgG peroxidase (Amersham). Colour development was stopped after 20-30 min by rinsing the membranes with distilled water.

The staining specificity of antibody 15F6 was confirmed by repeating the above procedure with the antibody pre-incubated with aa 497-507-BSA conjugate (100µl antibody and 100µl conjugate in 800µl PBS, pre-incubated overnight at 4°C). A negative control consisted of the antibody pre-incubated with aa 256-275-BSA.

2.17 Preparation of λgt11 Lysates

Liquid lysates from the immuno-positive clones isolated from the MCF-7 cDNA library were used for DNA preparation. These were produced by adding 100µl log phase *E.coli* Y1088 cells and approximately 10^8 PFU λ bacteriophage to 10 ml LB containing 10 mM $MgSO_4$, 0.4% maltose and 50µg/ml ampicillin. Cultures were grown at 37°C in a shaking incubator until the completion of lysis, usually after 6-8 h. Cultures showing no sign of lysis after 3 h were supplemented with extra phage. 100µl of chloroform was added to each culture for the last 15 min of incubation in order to aid cell lysis.

2.18 Preparation of λgt11 DNA by Polymerase Chain Reaction (PCR)

Lysates were subjected to PCR using custom primers, synthesized by Mr Howard Potter, Christchurch School of Medicine, Christchurch, New Zealand. The design of the forward primer (5' AATTAACCCTCACTAAAGGGGGTGGCGACGACTCCTGGAGCCCG 3') was based on a combination of the T3 universal primer sequence and the λgt11 forward primer sequence. GC content and annealing temperature were calculated at 59% and 75°C respectively. The reverse primer (5' GTAATACGACTCACTATAGGGCTTGACACC AGACCAACTGGTAATG 3') incorporates the T7 universal primer sequence and the λgt11 reverse primer sequence, with a GC content and annealing temperature of 45% and 70°C respectively.

Reactions were set up according to instructions provided with the ELONGase kit (Life Technologies) and run on a Hybaid Omnigene thermal cycler. Templates were denatured (94°C, 2min) and subjected to 30 cycles of denaturation (94°C, 30 sec), annealling (50°C, 15 sec) and extension (72°C, 45 sec). PCR products used for sequencing were subsequently purified (High Pure PCR Product Purification Kit, Boehringer Mannheim) and visualised by gel electrophoresis using 1% agarose gels and λ *pst* markers obtained from the Cytogenetics and Molecular Oncology Unit, Christchurch School of Medicine, Christchurch, New Zealand.

2.19 Restriction Digestion and Isolation of Insert DNA

Inserts were digested with *EcoRI* (37°C, 65 min, Life Technologies) and visualised on 1% agarose gels. DNA fragments were isolated from agarose gels by centrifugation of gel slices through a sterile siliconised glass wool column. To construct such a column, the small end of a 1 ml pipette tip was cut off and glass wool lightly packed inside the opening. DNA fragments to be used as probes or for cloning were placed on top of the glass wool and the tip placed inside a 1.5 ml microcentrifuge tube.

The microcentrifuge tube was then centrifuged at 14000 RPM for 15 min, and the fluid collected in the bottom of the tube was transferred to a fresh tube. The siliconised glass wool containing the compressed gel slice was discarded. The DNA was precipitated from the solution by adding 0.1 volumes of 3M sodium acetate (pH 5.2) and two volumes of ice cold ethanol and storing at -70°C for 20 min or until frozen. The precipitated DNA was

collected by centrifugation at 14000 RPM for 10 min, and washed twice with 70% ethanol. The DNA was then air-dried and resuspended in a small volume of TE buffer (5-10µl) and stored at -20°C until use.

2.20 Sequencing

Purified PCR products were quantified by comparison to molecular mass standards and sequenced by cycle-sequencing using a LI-COR Model 4000L sequencer, the SequiTherm Long-Read Cycle Sequencing Kit (Epicentre Technologies) or Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham) and T3 and T7 fluorescent primers (LI-COR). PCR-sequencing was carried out according to instructions supplied with each kit.

Briefly, a mixture containing template DNA (50-250 fmol), labelled primer (1µl), 10x sequencing buffer (2.5 µl), thermostable DNA polymerase (1µl) and deionised water (to bring volume up to 17 µl) was made up and 4 µl added to each of four 0.5 ml microcentrifuge tubes containing the ddNTPs G, A, T, and C, on ice. After the addition of one drop of mineral oil to each tube and brief centrifugation to separate the oil from the reaction mixture, templates were denatured (92°C, 2 min) and subjected to 30 cycles of denaturation (92°C, 30 sec), annealling (50°C, 15 sec) and extension (70°C, 15 sec). Stop solution (4µl) was added to each tube and 1.5 µl loaded onto the sequencing gel. Several sequencing reads in both directions were performed to eliminate sequencing errors and ambiguities. All sequencing was performed at the Cytogenetics and Molecular Oncology Unit, Christchurch School of Medicine, Christchurch, New Zealand.

2.21 ^{32}P Labelling, Plaque Lift and Library Screening

One of the inserts (3B) was labelled with ^{32}P-dNTP using the RTS RadPrime DNA Labelling System (Life Technologies). In order to examine sequence identity across clones, a plaque lift was done. 10µl of each lysate (10^7 FPU/ml) was spotted onto an LB plate with 0.5% agarose overlay containing 100µl Y1090 cells, and incubated for 8 h at 37°C. A negative control (consisting of a 260 bp hER cDNA) was included. A nitrocellulose membrane was placed on the plate for 1 minute, after which the membrane was transferred

to denaturation solution for 30 seconds followed by neutralisation solution, and finally 2XSSC. DNA was fixed in a UV Stratalinker.

The membrane was prehybridised at 65°C for 2 h in modified Church Buffer (Church, 1984), containing 500µl of salmon sperm DNA. Subsequent addition of the probe was followed by an overnight incubation at 65°C. Non-specific and unbound probe were removed during three 15-30 minute washes in 2 X SSC (1x) and 0.1 X SSC containing 0.1% SDS (2x). The membrane was exposed on Kodak X-OMAT AR film for 5 h at -80°C and developed at Radiology Services, Christchurch Hospital, Christchurch, New Zealand.

For library screening, the MCF-7 cDNA library was plated out on four 17 cm x 22 cm plates at a density of approx. 5 x 10^5 FPU/plate. After 6-7 h, the plates were overlaid with Hybond-N+ membrane (Amersham) and processed as above. Exposure times varied from 3-8 days depending on age of probe and number of previous uses.

2.22 Bioinformatics

Computational analysis of cDNA sequences was performed using BLAST (Basic Local Alignment Search Tool), accessed via e-mail (<blast@ncbi.nlm.nih.gov>) at the National Center for Biotechnology Information (NCBI). BLAST represents a family of programs which utilise algorithms in order to detect similarity between the submitted or query sequence and sequences present in the nucleotide or protein databases (Altschul *et al.*, 1990).

BLASTN searches against a non redundant nucleotide database were used to check for matches to previously identified human genes. BLASTX searches against a non redundant protein database were used to translate the obtained cDNA sequence into the six possible reading frames and check these for homology to known proteins. Expressed Sequence Tag (EST) entries were retrieved from the EST database (dbEST). This database contains over 343,000 expressed sequences (cDNA or mRNA) for which no information is available besides sequence (ie random cDNAs and end sequences). This corresponds to a significant proportion of all human genes and constitutes and invaluable tool for the molecular and functional characterisation of human genes. Sequences isolated from this database were

resubmitted in order to obtain overlapping ESTs (EST walking). Searches were repeated on a regular basis (every four to eight weeks).

Translation of the 260 bp sequence into all six possible reading frames was done by using GCG software.

2.23 Epitope Definition

Based on sequence translation data (fig 27), it was speculated that the epitope, or antigenic determinant, recognised by antibody 15F6 was likely to be contained within open reading frame +1 (ORF+1). Similarity to hER aa 497-507 (amino acid identity and charge similarity) was used as the criteria for selecting regions to be synthesised as peptides and tested by ELISA (Figure 6).

Peptide 1 Peptide 2

PATPTGQQQSQHGGSHPAPSPVQHHQHQAAQALHLASPQQQSAIYH

Peptide 3

AGLAPTPPSMTPASNTQSPQNSFPAAQQTVFTIHPSHVQPA

Figure 6 The translated 260 bp sequence in open reading frame +1 (ORF+1). The areas highlighted were selected for peptide synthesis in order to define the epitope of antibody 15F6.

Stock solutions of the peptides were prepared (100µg/ml in PBS) and used to create a series of dilutions (100, 50, 25, 12.5, 6.25 and 3.125µg/ml, in PBS). 100µl/well of each dilution was added to a microtitre plate and incubated overnight at 4°C. Following blocking (150µl/well of PBS assay buffer, 30 min), the plate was washed with wash buffer (4x) and antibody 15F6 (1:20 in PBS) was added for a 2 h incubation at room temperature. The plate was washed (4x), and 100µl/well of second antibody was added (goat antimouse Ig peroxidase, 1:1000 in PBS) for a 30 min incubation period. After a final washing (4x), substrate was added (100µl/well). Colour development was stopped by the addition of 100µl/well 1.25 M H_2SO_4. The absorbance was read at 492 nm.

The above experiment was repeated with peptide 2; in this case a 100μg/ml stock solution containing 0.1% acetic acid was prepared in order to improve the solubility of the peptide. Dose-response curves using the peptides and peptide conjugates were also generated according to the methodology listed in section 2.6.

Results

3.1 Dose-response Curves

Thirty antibody-secreting hybridomas which recognised the hER a.a.497-507-BSA conjugate were identified by ELISA. Eight of these gave good dose-response curves (DRCs) over the range of peptide/peptide conjugates tested (0-1mg/ml). Further dilution of the hybridoma supernatants preserved the DRCs for four of these (1F1, 1F2, 14F11, 10B7). Dilution of the hybridoma supernatants did not always result in obtaining more sensitive DRCs; in some cases dose-sensitive response was reduced or lost. This was the case for supernatants 1C1 and 9F10 respectively (data not shown). Hybridoma supernatant 15F6 did not generate a DRC, either undiluted or diluted, but is included for comparison (Figure 7).

DRCs, using 15F6 and increasing doses of hER aa 497-507 peptide and peptide conjugates with BSA and KLH as controls are shown in Figure 8. The BSA-peptide conjugate exerts the strongest competitive binding influence over antibody 15F6, with the KLH-conjugate and unconjugated peptide having a lesser, but similar influence in terms of peptide concentration. BSA and KLH alone do not affect antibody binding to the coated plate.

3.2 Binding Studies

Binding studies revealed that none of antibodies were able to immunoprecipitate the native hER from 16α-iodo-oestradiol labelled cytosols above control levels (data not shown).

3.3 Immunological Staining

Immunocytochemistry, using fixed MCF-7 cells and hybridoma supernatants identified one supernatant (15F6) showing predominantly nuclear staining (Figure 9). Other supernatants gave either no staining, cytoplasmic staining (Figure 10) or a mixture of cytoplasmic and nuclear staining, with varying intensities. Immunofluorescent staining tended to give less consistent staining patterns, however, nuclear staining with antibody 15F6 was observed.

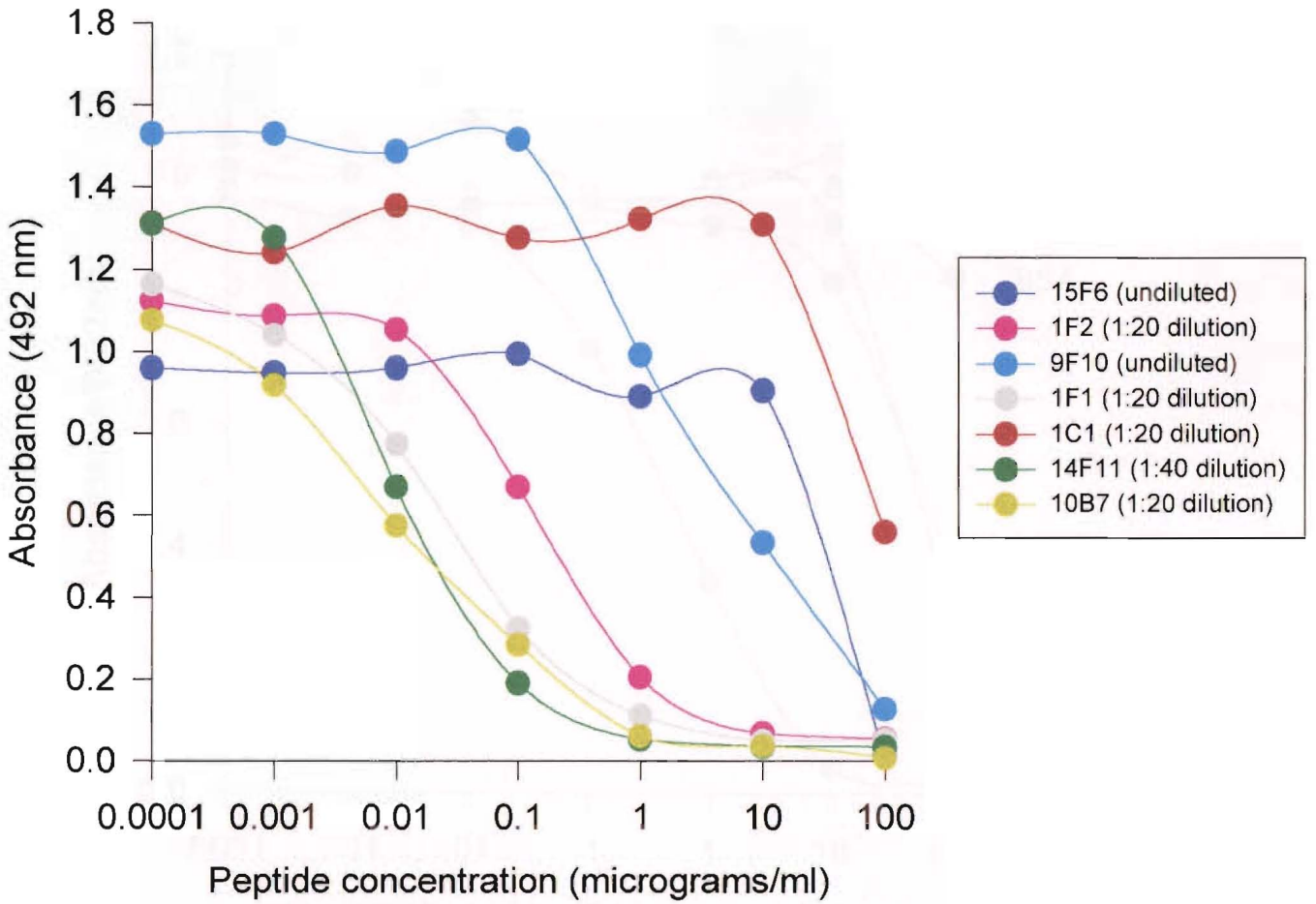

Figure 7 Dose-response curves of a selection of monoclonal antibodies raised against hER aa 497-507. For each curve, antibody was added to microtitre plates coated with hER aa 497-507-BSA conjugate, along with increasing doses of peptide (hER aa 497-507).

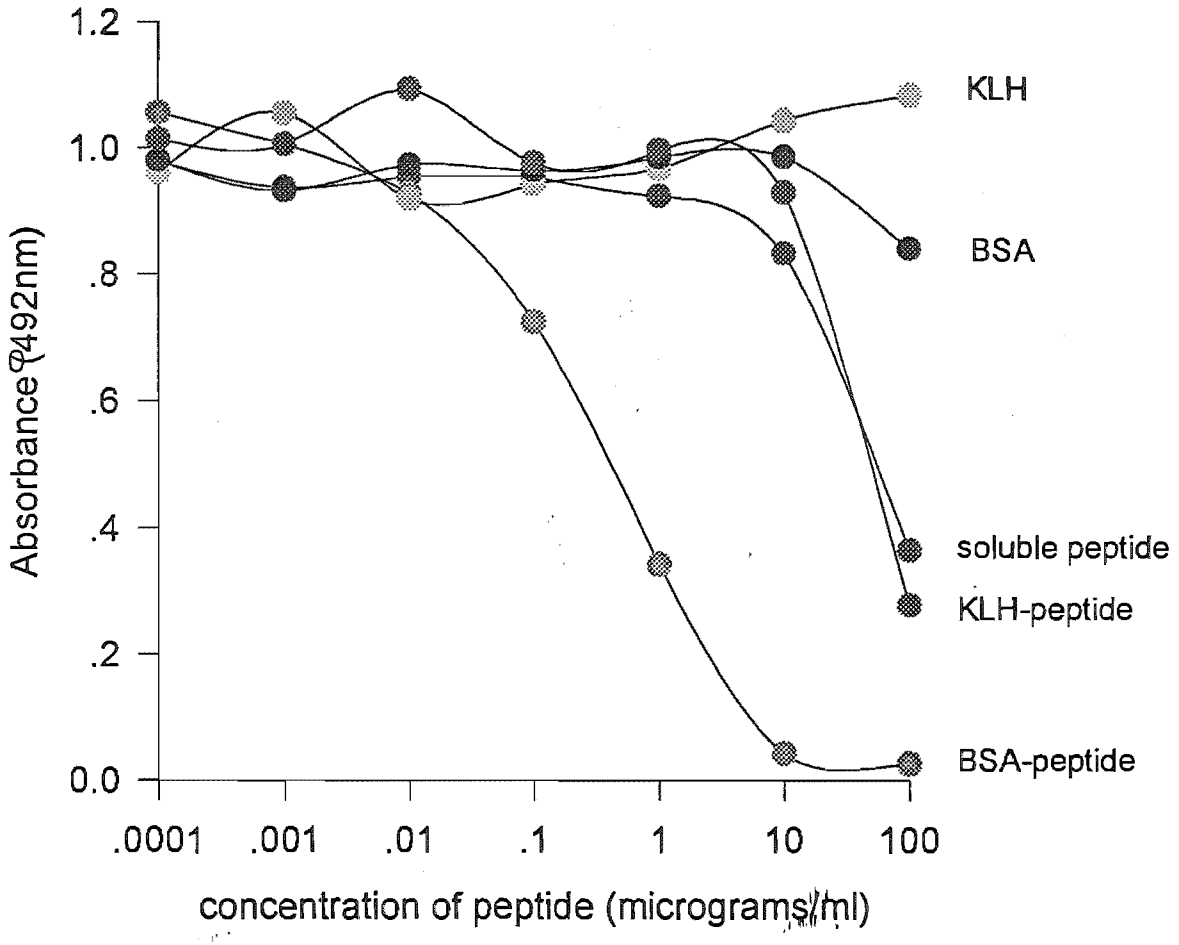

Figure 8 Dose-response curves generated by ELISA using antibody 15F6 (1:10 dilution). The antibody was added to hER aa 497-507-BSA coated microtitre plates, along with increasing doses of antigen (peptide or peptide conjugates). BSA and KLH were included to control for antigenicity generated by the conjugation of the peptide to the carrier protein.

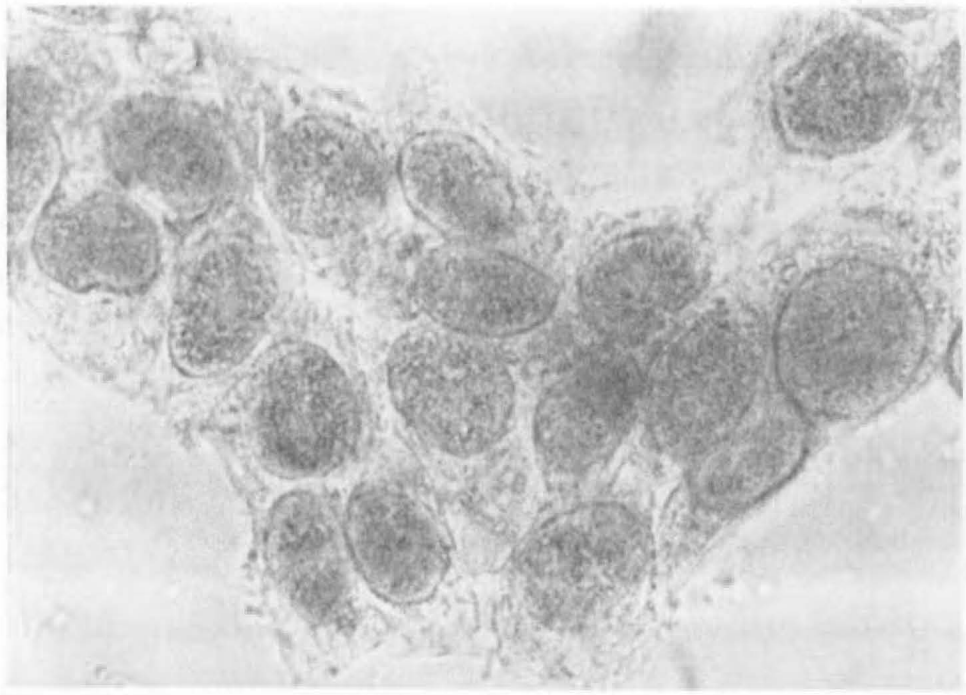

Figure 9. Immunoperoxidase staining of MCF-7 cell nuclei with antibody 15F6.

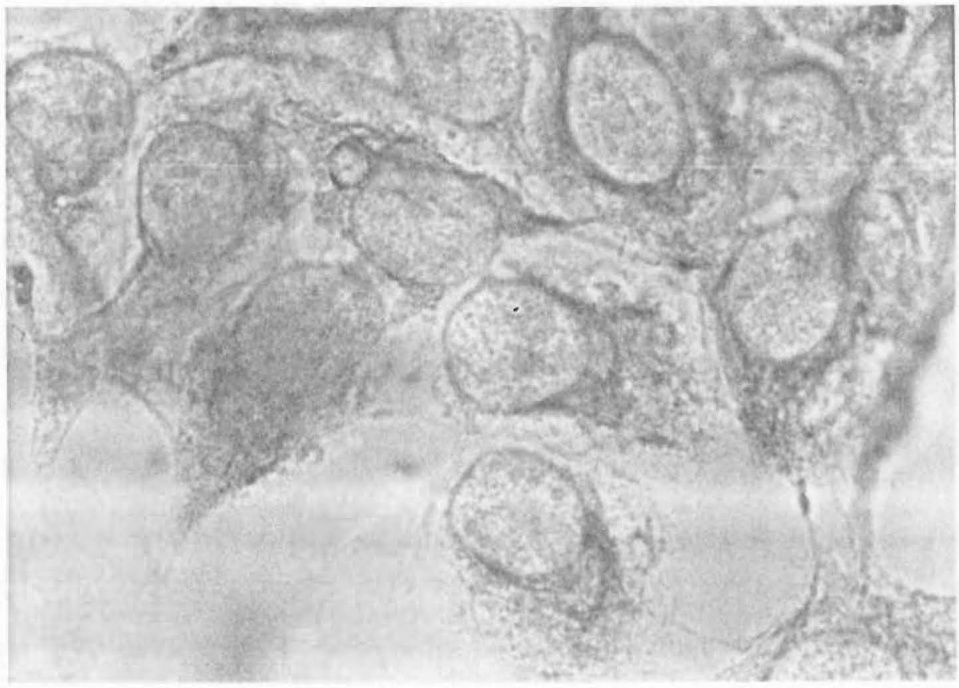

Figure 10. Immunoperoxidase staining of MCF-7 cell cytoplasm with antibody 9B3.

The immunoperoxidase staining observed with antibody 15F6 could be completely abolished by pre-incubation of the antibody with the soluble peptide a.a.497-507 (10 mg/ml), or a.a.497-507-BSA conjugate (1 mg/ml), and significantly reduced by pre-incubation of the antibody with a.a.497-507-KLH conjugate (1mg/ml) or the soluble peptide (1mg/ml). Staining was unaffected by pre-incubation of the antibody with either hER a.a.256-275-KLH (1 mg/ml) or a.a.256-275 (1 or 10mg/ml) (Figure 11).

Antibody 15F6 also stained the nuclei of two human breast cell lines which do not express the hER (MDA-MB-330 and MDA-MB-231), the human breast cell line T47D, and human liver cell line Hep2G. Staining of the hER negative cell lines could be abolished by pre-incubation of the antibody with the soluble peptide and peptide conjugates (Figures 12 and 13). The staining of human tissue sections reveal the antigen to be present in the nuclei of keratocytes in skin (Figures14-16) and tubule and luminal endothelial cells of the kidney (Figures17-19).

3.4 Western Blot Analysis

Antibody 15F6 consistently identified a 120 kD protein in solubilized whole cells and cytosol extracts prepared from these cell lines (Figure 20).

3.5 Isotyping of Antibody 15F6

Isotyping revealed 15F6 to be an IgM κ antibody.

3.6 ELISA and Western Blotting of the Purified Protein Extract

The protein extract obtained by affinity chromatography demonstrated immunoreactivity with antibody 15F6 by ELISA (Figure 21). Subjecting the extract to SDS-PAGE and staining the gel with Coomassie Blue allowed for the detection of several protein bands in addition to the (presumed) antigen (data not shown). Western blotting using antibody 15F6 as a probe failed to generate a signal.

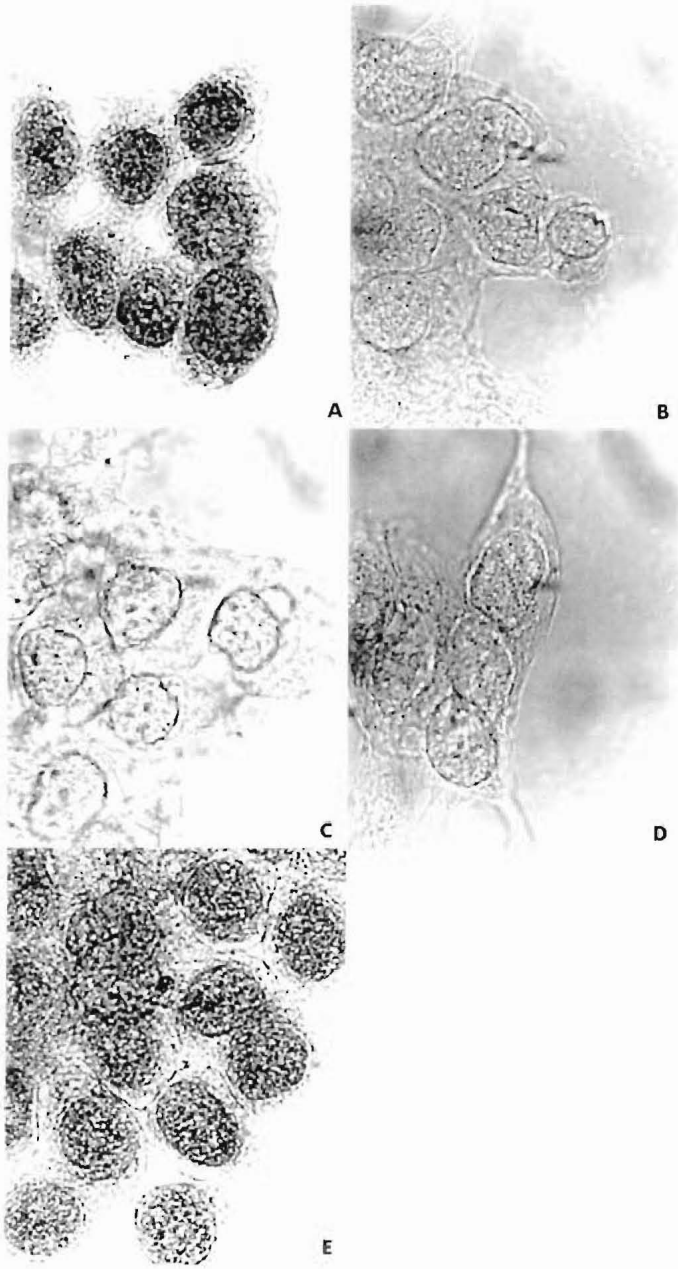

Figure 11. Immunoperoxidase staining of MCF-7 cells using antibody 15F6.
(a) Staining with 15F6.
(b) 15F6 pre-incubated with hER a.a.497-507-BSA (1 mg/ml).
(c) 15F6 pre-incubated with hER a.a.497-507-KLH (10 mg/ml).
(d) 15F6 pre-incubated with hER a.a.497-507 (10mg/ml).
(e) 15F6 pre-incubated with hER a.a.256-275 (10mg/ml).

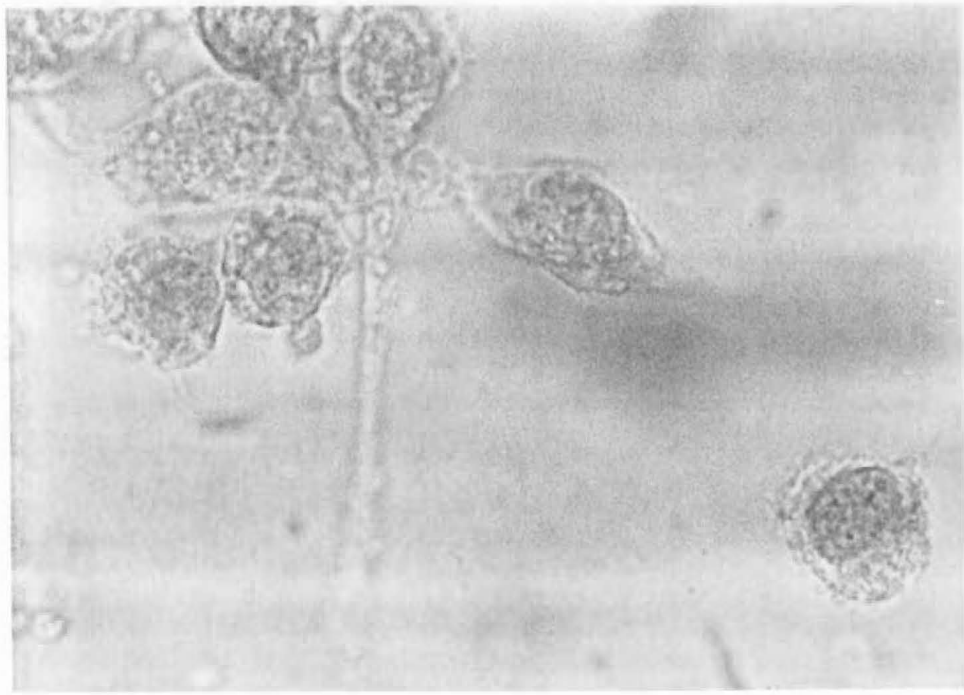

Figure 12. Immunoperoxidase staining of MDA-MB-231 cell nuclei with antibody 15F6.

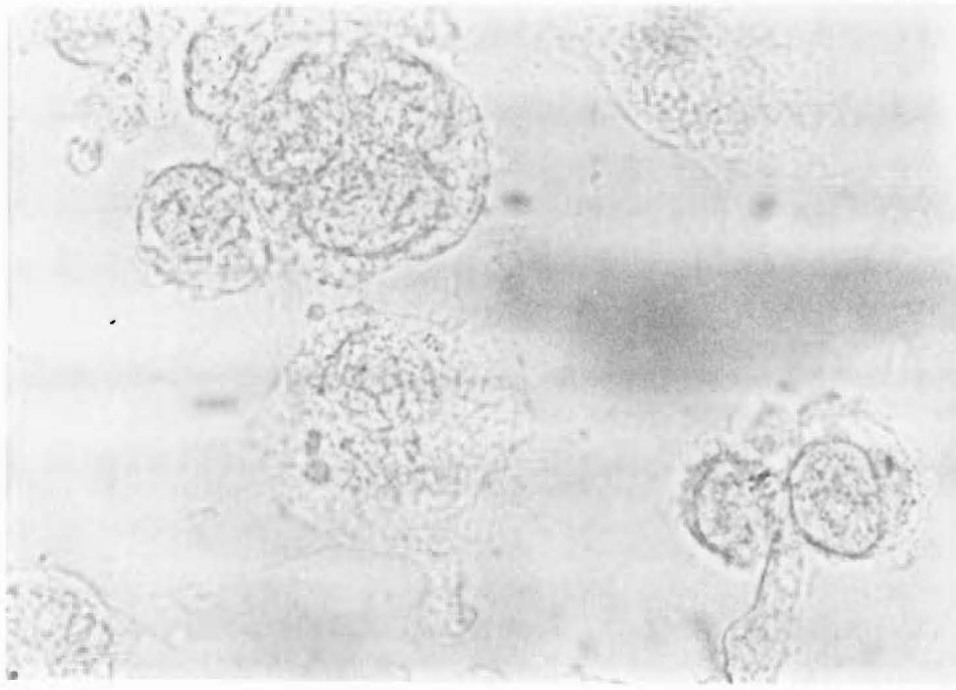

Figure 13. Immunoperoxidase staining of MDA-MB-231 using antibody 15F6 pre-incubated with hER aa 497-507 (10mg/ml)

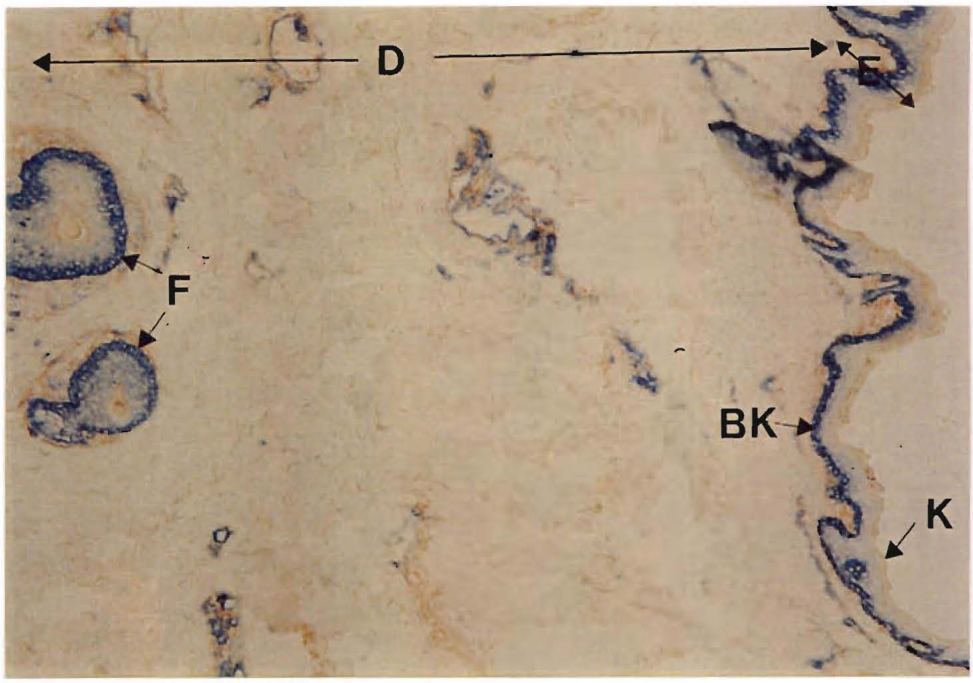

Figure 14. Photograph of human skin section showing location of basal keratinocytes **(BK),** hair follicles **(F)**, dermis **(D)**, epidermis **(E)** and associated keratin **(K)**. All skin sections are double labelled with antibodies VM-2 (Fast Blue substrate) and 15F6 (AEC substrate) (x 100).

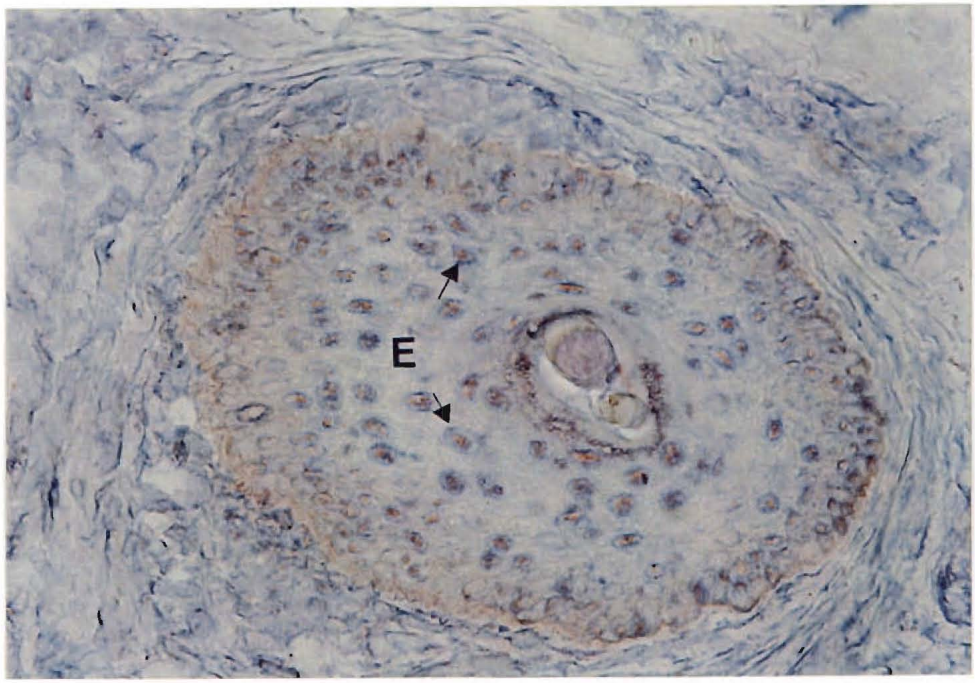

Figure 15. Photograph showing nuclear staining of epidermal cells **(E)** surrounding hair follicle with antibody15F6 (AEC substrate) (x 400).

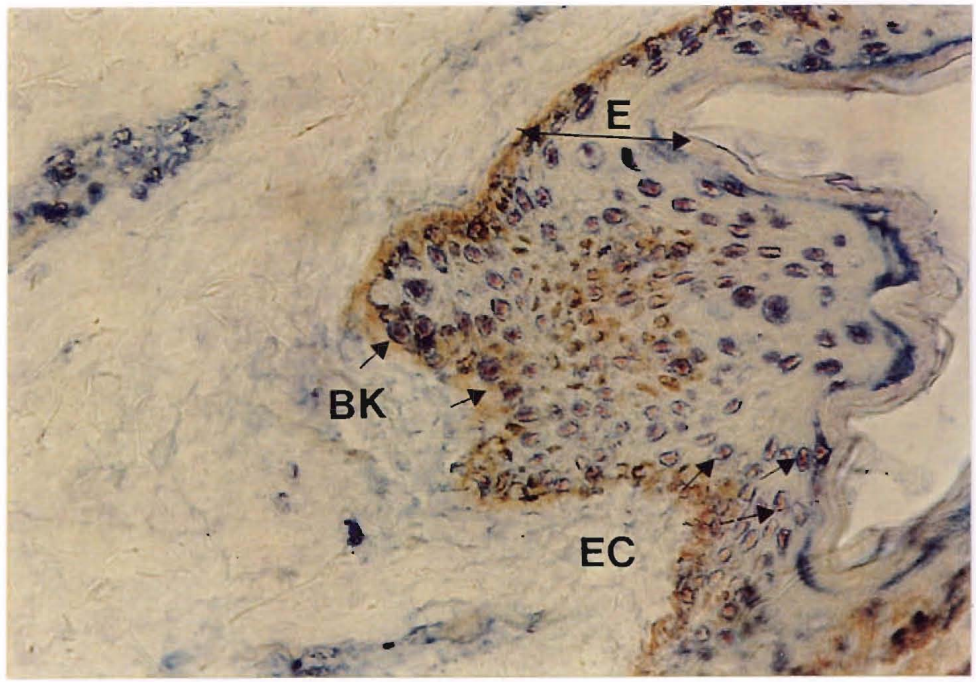

Figure 16. Photograph showing detailed nuclear staining of all layers of epithelial cells **(EC)** within the epidermal layer **(E)** using antibody 15F6 (Fast Blue substrate). Basal keratinocytes **(BK)** are stained using antibody VM-2 (AEC substrate)(x 400).

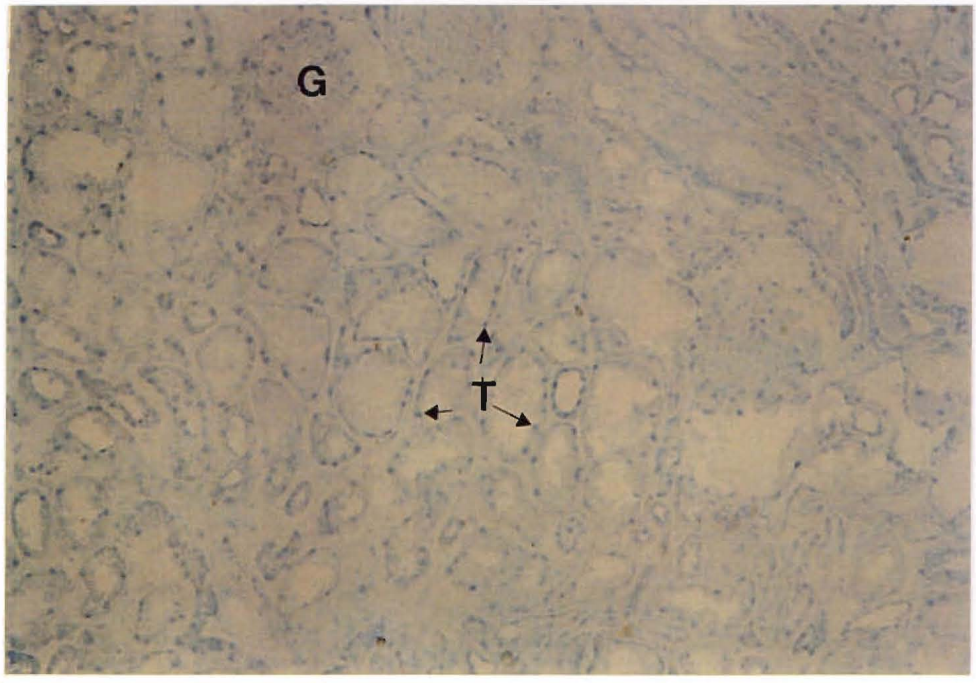

Figure 17. Photograph of human kidney section showing nuclear staining of tubule epithelial cells **(T)** using antibody 15F6 (Fast Blue substrate). Nuclear staining of glomeruli **(G)** is also visible in this section (x 100).

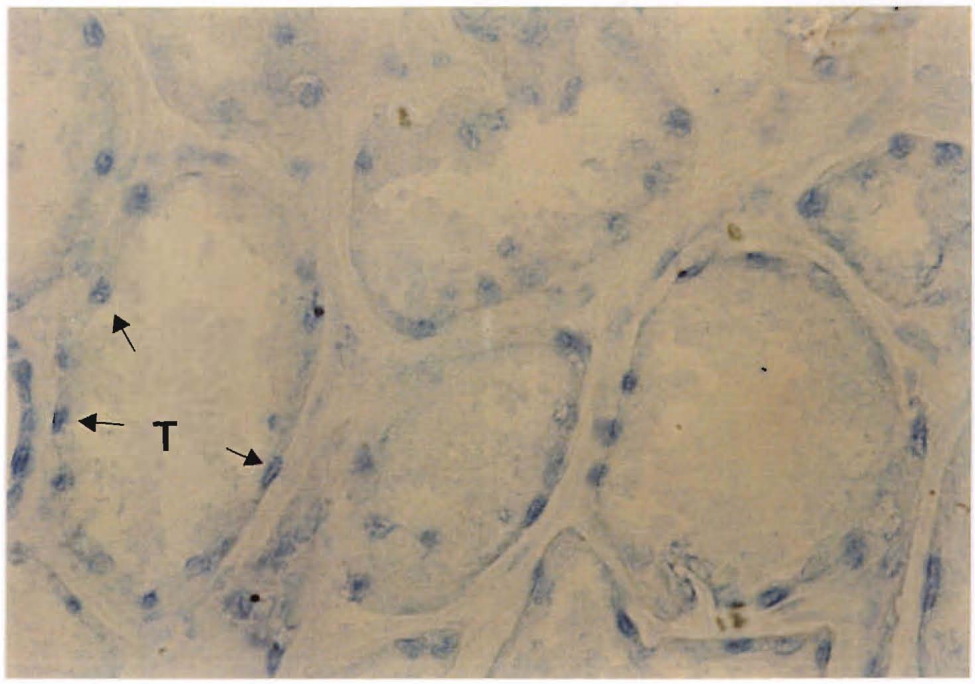

Figure 18. Photograph showing detail of nuclear staining of kidney tubule epithelial cells **(T)** using antibody 15F6 (Fast Blue substrate)(x 400).

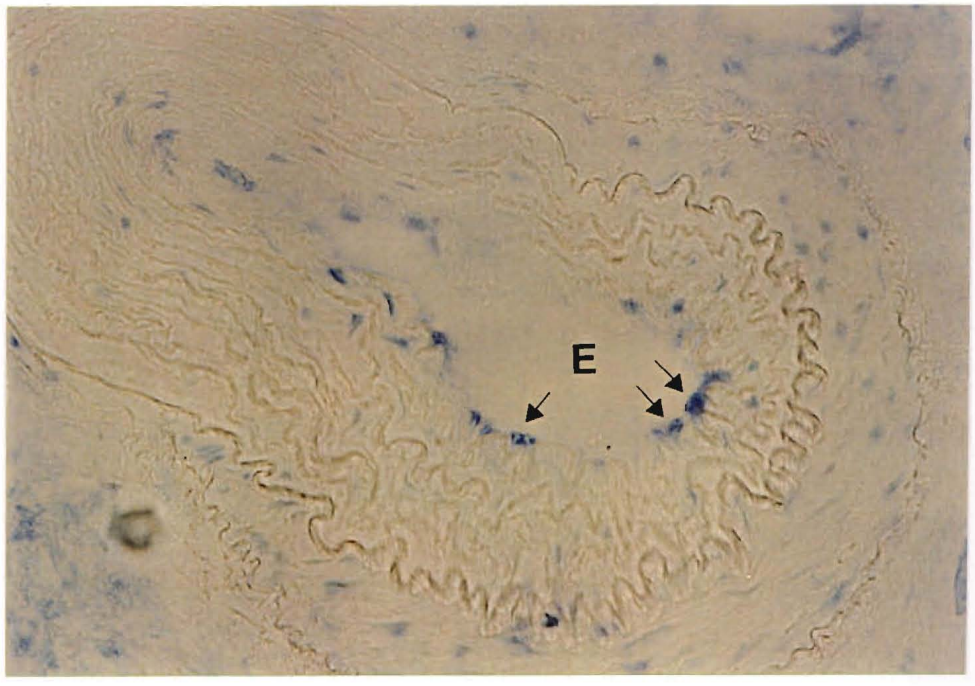

Figure 19. Photograph showing nuclear staining of luminal endothelial cells **(E)** in arteriole of kidney using antibody 15F6 (Fast Blue substrate)(x 400).

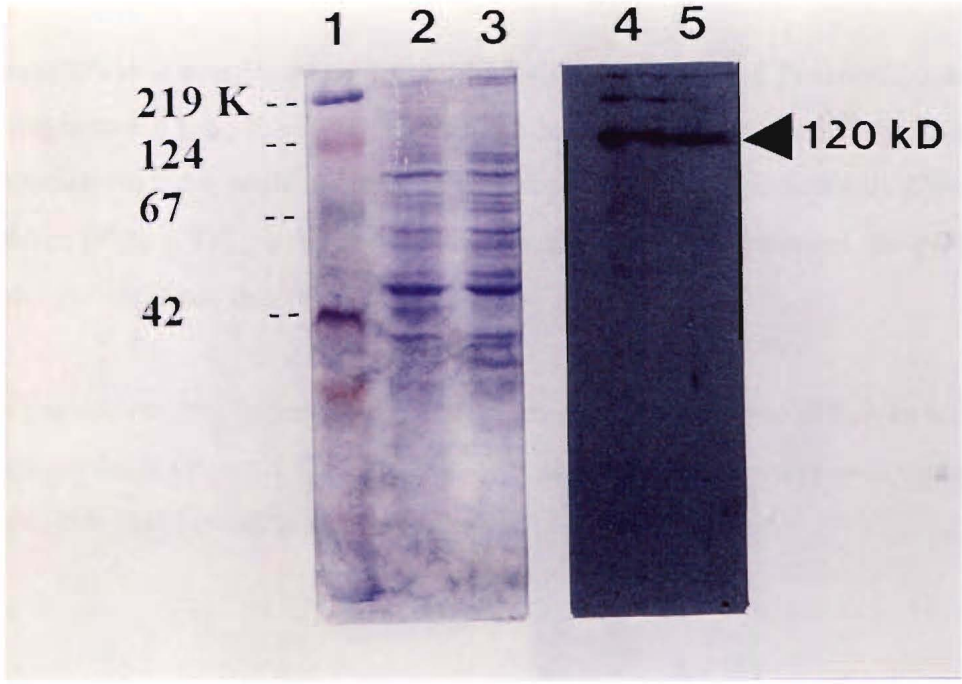

Figure 20. SDS-PAGE Western blot of cytosol extracts from breast cancer cell lines.
Lane 1; BioRad Kaleidoscope molecular weight markers.
Lanes 2 and 4; MCF-7 cytosol stained with Coomassie Blue and 15F6 respectively.
Lanes 3 and 5; MDA-MB-231 cytosol stained with Coomassie Blue and 15F6 respectively.

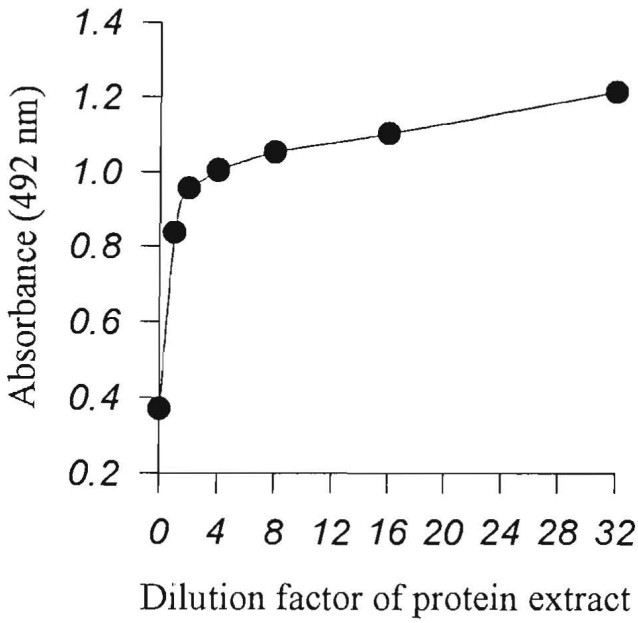

Figure 21 ELISA competition experiment using monoclonal antibody 15F6 with varying dilutions of the protein extract on a hER aa 497-507-BSA coated assay plate.

3.7 Immunodiffusion and Immunoelectrophoresis of the Purified Protein Extract

Immunodiffusion between the protein extract and anti human serum gave a strong positive immunoprecipitation reaction, while no reaction was observed with either antibody 15F6 or anti ceruloplasmin (Figure 22). Immunoprecipitation did also occur between the protein extract and anti IgM (data not shown).

Application of immunoelectrophoresis to the extract revealed the presence of human serum albumin as a contaminant (Figure 23). Unfortunately, the impurities present in the extract rendered it unsuitable for N-terminal sequence analysis.

3.8 Antibody screening of the MCF-7 cDNA library

Expression-linked screening of the MCF-7 cDNA library with antibody 15F6 resulted in the isolation of 22 immunoreactive clones. Re-screening of the plaque-purified clones with the more specific peroxidase substrate resulted in the elimination of 12 clones. Antibody specificity for the remaining ten clones was confirmed by an antibody-preincubation experiment, in which antibody staining was abolished by pre-incubating 15F6 with the a.a.497-507-BSA conjugate (Figure 24).

Amplification by PCR, restriction enzyme digestion and agarose gel electrophoresis allowed for insert size determination; eight of these inserts were approx. 260 bp, one insert was approx. 100 bp, while the remaining insert did not give a PCR product. Sequence identity between at least eight of the inserts was confirmed by hybridisation with a ^{32}P-labelled insert as a probe. Five inserts were sequenced and found to be identical (data not shown).

3.9 Bioinformatics

Sequence was submitted to the BLASTN (nucleotide) and BLASTP (protein) databases for homology analysis. The results of the BLAST searches confirm the novelty of this sequence; no gene or protein sequences were found to have sufficient homology (100% or slightly less depending on the presence of sequencing errors) to the complete submitted query sequence.

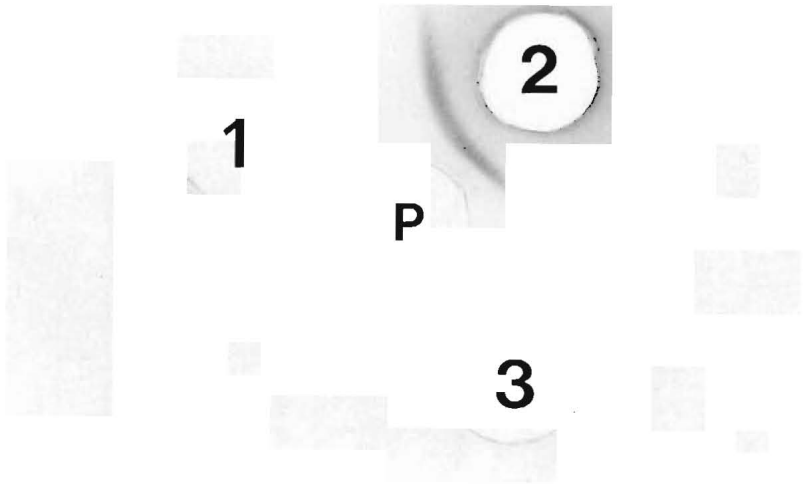

Figure 22. Immunodiffusion of the protein extract (P) against antibody 15F6 (1), anti human serum (2), and ceruloplasmin (3). The precipitin line between the extract and anti human serum indicates immunoprecipitation.

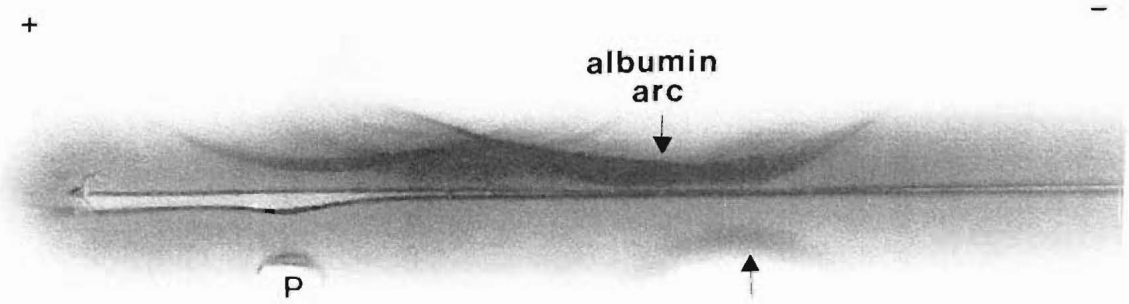

Figure 23 Immunoelectrophoresis of the protein extract. Immunoprecipitation between anti human serum and serum results in the formation of a series of precipitin arcs, acting as a reference for the characterisation of contaminant protein(s).

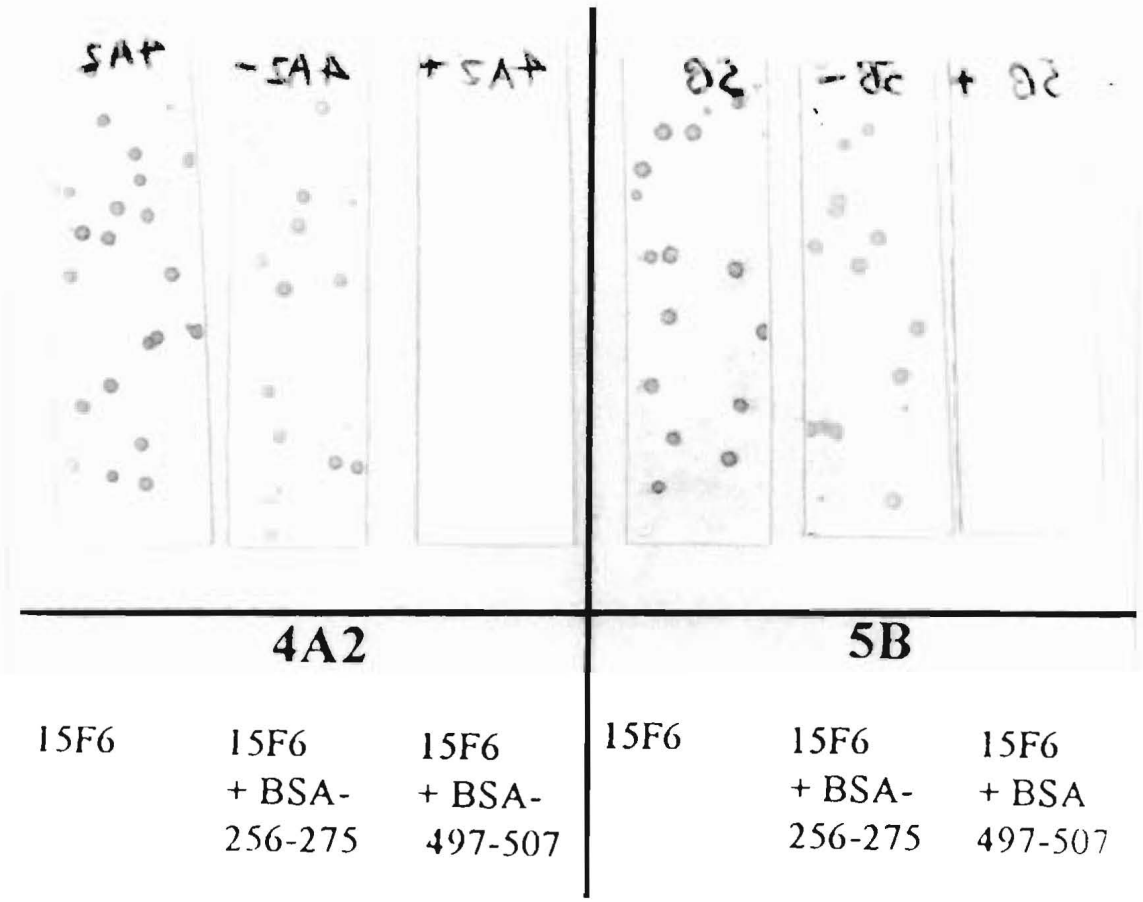

Figure 24. Expression-linked screening of the MCF-7 cDNA library (refer to Methods section for details of procedure). Immunoreactivity between expressed fusion protein from plaque-purified clones 4A2 and 5B and antibody 15F6 can be seen on the nitrocellulose strips. Specificity of this interaction was demonstrated by pre-incubation of the antibody with hER aa 497-507 BSA conjugate (1mg/ml) prior to staining. Pre-incubation of the antibody with an unrelated peptide (hER 256-275-BSA conjugate) was included as a negative control and did not abolish staining.

A low degree of homology to parts of several non-human genes and mRNA sequences was detected in the BLASTN database search. These include an alkaline protease gene from *A.chrysogenum* (48/75 positives, 64%), a rat pyruvate kinase gene (33/43 positives, 76%), a voltage-gated chloride channel mRNA from *T.california* (39/58 positives, 67%) and a cyclase associated protein mRNA from *D.discoideum* (37/54 positives, 68%). Homology to human DNA sequence between markers DXS6791 and DXS8038 on chromosome X was also found (35/49 positives, 71%)(Figure 25). The BLASTP search yielded results with a similar pattern; where homology to the query sequence was reported, it was only over very short stretches of amino acids and not complete.

Submitting the sequence to the "est" database (dbEST) using the BLASTN program resulted in the isolation of several expressed sequence tags with partial or complete overlap with the query sequence. These were resubmitted to the database in order to isolate additional overlapping clones. This process of EST walking allowed for the construction of a 1.6 kb contig map (Fig 26).

The largest of the ESTs is a 1.2 kb fetal brain cDNA clone and contains a TATAA box. The origin of these ESTs (ie the tissue from which their respective cDNA library was constructed) has revealed the antigen to be present in brain, heart, liver, lung, spleen, colon, ovary, uterus and placenta (Table 2).

Translation of the 260 bp sequence into the six possible reading frames (ORFs) yielded only one reading frame which did not contain stop codons (Figure 27). This ORF contained several potential epitopes (ie regions with some homology to hER aa 497-507) and was therefore assumed to be the most likely reading frame. On the basis of this selection, three regions were chosen for peptide synthesis (section 2.3/Figure 6).

3.10 Plaque Lift and Library Screening

At least eight of the clones isolated from the MCF-7 cDNA library hybridise to the ^{32}P-labelled probe under the conditions used for the plaque lift, leading to the assumption that they are likely to be identical. Re-screening of the MCF-7 library with the ^{32}P-labelled probe was unsuccessful (data not shown).

```
Sequences producing High-scoring Segment Pairs:                Score  P(N)       N

dbj|D00923|APEALP  A.chrysogenum gene for alkaline protea...    132  0.40       1
gb|M17089|RATPKRL2 Rat pyruvate kinase gene, exons 3-9.         125  0.86       1
emb|X60433|TCVGCC  T.californica mRNA for voltage-gated c...    119  0.998      1
emb|Z76735|HS24608 Human DNA sequence from PAC 24608, bet...    119  0.999      1
gb|U43027|DDU43027 Dictyostelium discoideum cyclase assoc...    117  0.99990    1


>dbj|D00923|APEALP A.chrysogenum gene for alkaline protease, complete cds.
           Length = 3152

  Plus Strand HSPs:

 Score = 132 (36.5 bits), Expect = 0.51, P = 0.40
 Identities = 48/75 (64%), Positives = 48/75 (64%), Strand = Plus / Plus

Query:    14 CCCCCACTGGACAGCAGCAAAGCCAACATGGTGGAAGTCATCCTGCACCCAGTCCTGTTC 73
             || | ||||  || || ||  ||||||||||     ||  ||  | | | |||||  ||
Sbjct:   904 CCTCAACTGCTCACCAACACCGCCAACATGGTCACCCTCCGCCGCCTCGCCGTCCTCCTC 963

Query:    74 AGCACCATCAGCACC 88
             || |||||  | ||
Sbjct:   964 GGCGCCATCCCCGCC 978
```

Figure 25 Example of information retrieved from a BLAST search using the BLASTN program. BLASTN compares a nucleotide query sequence against a nucleotide sequence database in order to obtain sequence alignments. Five partial alignments to the query sequence (260 bp sequence isolated from the MCF-7 cDNA library) were reported. Shown below are details of one of the alignments; in this case the low degree of homology (64%) between nucleotide 14-88 of the query sequence and nucleotide 904-978 of the *A. chrysogenum* cDNA encoding alkaline protease. Homology of 100% or slightly less (to allow for sequencing errors) must be obtained in order to make the assumption that the query sequence is equivalent to a reported gene sequence.

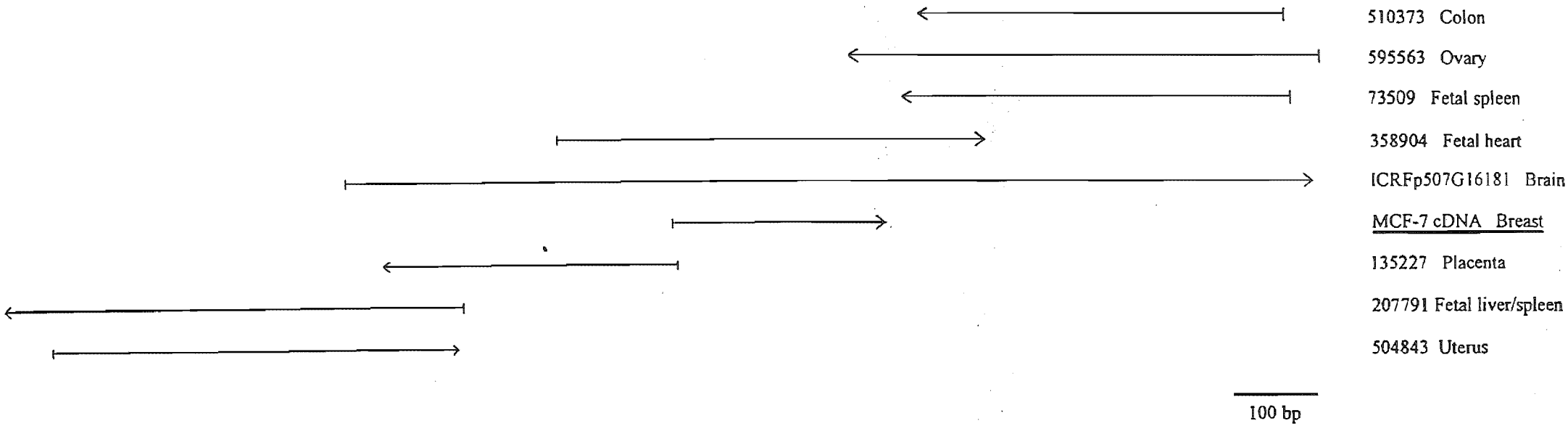

Figure 26. Contig map generated by EST walking. The 260 bp cDNA isolated from the MCF-7 library was submitted to dbEST in order to obtain sequence for available, overlapping clones. EST walking provides additional sequence data without the need to perform further library screening, thus saving considerable time and resources. In this case, EST walking also gives an indication of the tissue types in which this antigen is expressed.

A.

```
  1  CAGCTACCCC CACTGGACAG CAGCAAAGCC AACATGGTGG AAGTCATCCT
 51  GCACCCAGTC CTGTTCAGCA CCATCAGCAC CAGGCCGCCC AGGCTCTCCA
101  TCTGGCCAGT CCACAGCAGC AGTCAGCCAT TTACCACGCG GGGCTTGCGC
151  CAACTCCACC CTCCATGACA CCTGCCTCCA ACACGCAGTC GCCACAGAAT
201  AGTTTCCCAG CAGCACAACA GACTGTCTTT ACGATCCATC CTTCTCACGT
251  TCAGCCGGCG
```

B.

```
  1  EREKDGS*RQ SVVLLGNYSV ATACWRQVSW RVELAQAPRG KWLTAAVDWP
 51  DGEPGRPGAD GAEQDWVQDD FHHVGFAAVQ WG*LGI

  1  RLNVRRMDRK DSLLCCWETI LWRLRVGGRC HGGWSWRKPR VVNG*LLLWT
 51  GQMESLGGLV LMVLNRTGCR MTSTMLALLL SSGGS

  1  AG*T*EGWIV KTVCCAAGKL FCGDCVLEAG VMEGGVGASP AW*MADCCCG
 51  LARWRAWAAW C*WC*TGLGA G*LPPCWLCC CPVGV
```

C.

```
  1  ATPPTGQQQS QHGGSHPAPS PVQHHQHQAA QALHLASPQQ QSAIYHAGLA
 51  PTPPSMTPAS NTQSPQNSFP AAQQTVFTIH PSHVQPA

  1  QLPPLDSSKA NMVEVILHPV LFSTISTRPP RLSIWPVHSS SQPFTTRGLR
 51  QLHPP*HLPP TRSRHRIVSQ QHNRLSLRSI LLTFS

  1  SYPHWTAAKP TWWKSSCTQS CSAPSAPGRP GSPSGQSTAA VSHLPRGACA
 51  NSTLHDTCLQ HAVATE*FPS STTDCLYDPS FSRSA
```

Figure 27 Nucleotide and amino acid sequence of the 260 bp insert isolated by screening the MCF-7 cDNA library with antibody 15F6.

A. nucleotide sequence of strand assigned as 'plus' strand

B. possible amino acid translations of 'minus' strand (strand complimentary to **A**)

C. possible amino acid translations of 'plus'strand (**A**)

Stop codons in **B.** and **C.** are denoted by * and correspond to TAA, TAG or TGA.

Unique Identifier	Clone no.	Source	Length (bp)
emb|Z78331|HSZ78331	ICRFp507G16181	fetal brain	1159
gb|AA167312|AA167312	595563	ovary	569
gb|H59045|H59045	207791	fetal liver/spleen	557
gb|W94719|W94719	358904	fetal heart	520
gb|AA150956|AA150956	504843	pregnant uterus	493
gb|T55469|T55469	73509	fetal spleen	469
gb|AA053667|AA053667	510373	colon	443
gb|R32955|R32955	135227	placenta	363
gb|R12495|R12495	128289	fetal liver/spleen	363
gb|R10604|R10604	128875	fetal liver/spleen	278

Table 2 EST walking; details of expressed sequence tags isolated with the 260 bp sequence obtained in this study as the starting point.

3.11 Epitope Definition

For details of peptides selected for epitope definition experiments, refer to section 2.3/Figure 6.

Antibody 15F6 displays the greatest affinity for peptide 2 (Figure 28 A). The solubility of this peptide was extremely low (the peptide contains 11/25 hydrophobic residues), making it difficult to use under standard assay conditions. Alternative solvents (dimethyl-formamide, dimethylsulfoxide) did little to improve solubility; however, an increase in antibody binding can be seen when the peptide is dissolved in PBS containing 0.1% acetic acid (Fig 28 B). Dose-response curves, using peptide conjugates, show that the antibody has the greatest affinity for the peptide 2 conjugate (data not shown). It appears, therefore, that peptide 2 contains the epitope recognised by antibody 15F6.

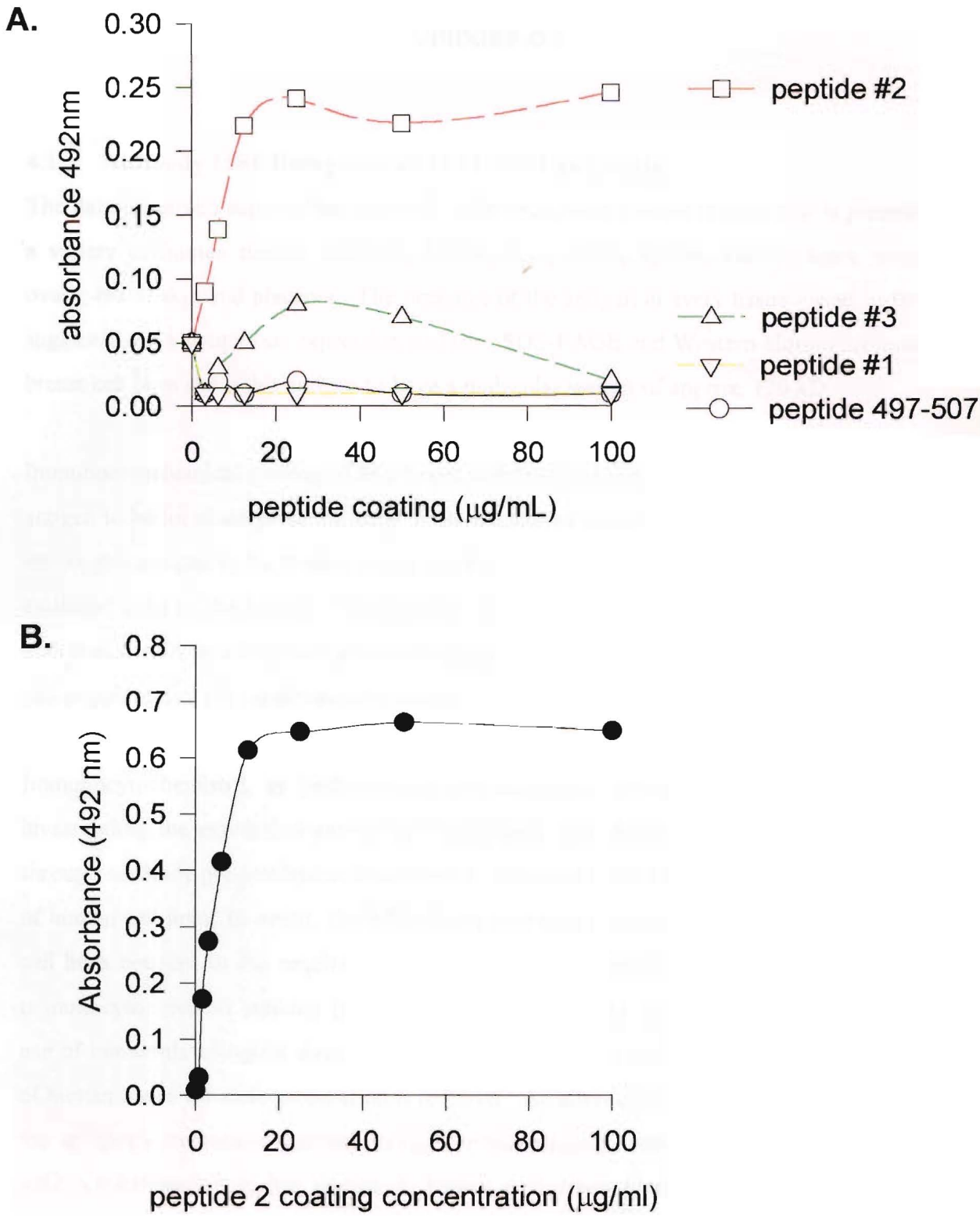

Figure 28. Epitope definition studies using ELISA.
A. Antibody 15F6 binding to peptides 1, 2 and 3 compared to binding of hER aa 497-507.

B. Antibody 15F6 binding to peptide 2 under enhanced peptide solubilisation conditions.

Discussion

4.1 Antibody 15F6 Recognises a 120 kD Nuclear Protein

The data presented suggest that antibody 15F6 recognises a novel protein that is present in a variety of human tissues, including breast, liver, colon, spleen, kidney, heart, uterus, ovary, brain, skin and placenta. The presence of the antigen in every tissue tested so far is suggestive of a ubiquitous expression pattern. SDS-PAGE and Western blotting of human breast cell lines show this antigen to have a molecular weight of approx. 120 kD.

Immunocytochemical staining of four breast cell lines and one liver cell line have shown this antigen to be localised predominantly in the nucleus of these cells. Immunological staining shows this antigen to be present in the nuclei of epithelial keratinocytes in skin and tubule epithelial cells of the kidney. Furthermore, the occurrence of nuclear staining of luminal endothelial cells in a nephritic arteriole suggests that the antigen is present in at least one cell population of the cardiovascular system.

Immunocytochemistry, as performed in this study, has proved to be a valid tool for investigating the expression pattern of this antigen, with staining specificity demonstrated through antibody pre-incubation experiments. It would be of interest to test a wider range of human cell lines, however, the difficulty in obtaining and maintaining a range of human cell lines coupled to the requirement that these be preferentially adherent cell lines make immunocytochemical staining (avoiding cytocentrifugation) an unattractive option. The use of immunohistological staining would circumvent these difficulties, however, a source of human tissue for slide preparation is required. An alternative approach to characterising the antigen's expression pattern through immunological staining would be to perform mRNA hybridisation studies, commonly known as Northern blotting.

4.2 Antibody 15F6 Does Not Recognise the Native hER

Of interest is the degree of specificity shown by antibody 15F6. The antibody did not precipitate the hER from 16α-iodo-oestradiol labelled breast cytosols, even under conditions which favour dissociation of hER dimers (Thole *et al.*, 1991). Western

blotting using this antibody failed to generate a signal in the region of the hER (67 kD). These two findings indicate that the antibody does not recognise the native hER.

4.3 Antibody 15F6 has a Degree of Specificity for the hER a.a.497-507 Region

While the antibody does not recognise the native hER, there is a low and quantitatively unimportant degree of recognition of the hER aa 497-507 region. Using ELISA, dose-response curves can be generated with the antibody and increasing doses of aa 497-507-BSA conjugate, and to a lesser extent aa 497-507-KLH and the unconjugated aa 497-507 peptide.

This data is supported by the immunohistochemistry data where nuclear staining could be blocked by pre-incubating the antibody with either aa 497-507-BSA, aa 497-507-KLH or the unconjugated peptide. Staining could not be blocked by pre-incubation of the antibody with similar concentrations of an unrelated peptide, hER aa 256-275 and its conjugates, aa 256-275-BSA and aa 256-275-KLH. It appears therefore that antibody 15F6 is specific for a region with some homology to hER aa 497-507.

It is possible that the conjugation of the peptide to the carrier protein (prior to immunization) induces a change in the biological epitope, resulting in a heterogenous population of conjugates with different orientations of peptide sequences. This would give rise to a diverse range of monoclonal antibodies. This is evident from the dose-response curves generated for each of the hybridoma supernatants. Of the 30 'positive' supernatants (ie displays reactivity with hER aa 497-507-BSA), only eight generated dose-response curves over the range of 0-1mg/ml soluble peptide, and seven of these had more sensitive dose-response curves than 15F6. However, none of these antibodies displayed nuclear staining with MCF-7 cells, or generated a signal on SDS-PAGE Western blotting using either MCF-7 cytosols or solubilized cells. Another possibility that could account for the diversity of the monoclonal antibodies produced is the presence of minor impurities in the immunising peptide.

4.4 Purification of the Protein

An attempt to purify this antigen by affinity chromatography was partially successful; while the protein extract displayed immunoreactivity with antibody 15F6 by an ELISA competition experiment, no signal could be detected on a Western blot. SDS-PAGE was used to check the protein extract for purity, and revealed the presence of several additional proteins. Some of the immunoreactive contaminants could be identified through the use of immunodiffusion and immunoelectrophoresis. These were found to include human IgM (probably from the IgM affinity column used in the purification) and albumin, respectively. Albumin is probably the component that accounts for the strong reactivity seen between the protein extract and anti human serum with immunodiffusion.

Immunoaffinity purification using monoclonal antibodies does not always work; cross-reactivity is a problem observed with a subset of monoclonal antibodies (Harlow & Lane, 1988). These antibodies bind to other antigens through shared epitopes. It is not possible to know whether this is occurring with the unidentified proteins in the extract. Proteins may also be co-purified with the antigen if the antigen is part of a complex under normal cellular conditions.

An interesting observation made regards the dark green colour of the protein extract, suggesting either contamination or the presence of a copper-binding protein. Immunodiffusion of the extract against anti ceruloplasmin antibody shows that this protein is not a member of the ceruloplasmins (principal carriers of copper in human plasma). A possible contaminant giving rise to this colour are the molybdate ions present in the TED buffer used during the purification procedure.

Although immunoreactivity between the extract and antibody 15F6 was shown by ELISA, no precipin line was generated by immunodiffusion. This may be a property of the antibody, as some antibodies do not form precipitating complexes under the conditions employed. Alternatively, the lack of signal may be due to the greatly reduced sensitivity of immunodiffusion compared to the ELISA.

Due to the failure to obtain enough antigen with sufficient purity, N-terminal sequencing was not performed. Increasing the number of cells used in the purification procedure

would increase the final yield of the antigen, however further purification procedures would have to be implemented in order to perform N-terminal sequencing.

4.5 Sequence Analysis Does Not Reveal the Identity of the Antigen

Submission of the DNA sequence obtained in this study to nucleotide (BLASTN) and protein (BLASTP) databases has not provided an identification for this antigen. The availability of ESTs provides some indication of the tissues which express the antigen as well as additional sequence. EST walking is a much cheaper, faster and less labour intensive means of obtaining sequence than repeat library screening. However, there are limitations on what can be done with the sequence; the relevant EST clones must be purchased and re-sequenced in order to provide accurate sequence before assumptions about the translated gene product can be made.

Using EST walking in combination with sequencing, repeat library screening, or a combination of both, could be used to derive the complete cDNA structure, including the untranslated regions (UTs) at the 3' and 5' ends of the cDNA, a TATA box, initiation and termination codons, polyadenylation signals, and a poly(A) tail at the 3'end. A TATA box was identified in one/two of the ESTs. Downstream from this site are several possible initiation codons (consisting of methionine residues).

With regard to the current study, ESTs have another potential use; they can be used as probes to define the chromosomal location of the respective gene. The 1.2 kb EST referred to in this study could be used for chromosomal localisation using fluorescence *in situ* hybridisation (FISH). *In situ* hybridisation provides the most direct way to study the chromosomal localisation of DNA sequences (Lichter and Cremer, 1992). Other techniques which can be used for this purpose include somatic cell hybrid and radiation hybrid mapping, and identification of previously mapped yeast artificial chromosome (YAC) clones (Berry *et al.*, 1995; Banfi *et al.*, 1996).

FISH allows for determination of location, size and number of specific DNA sequences in mammalian cells, and involves hybridisation of a selected DNA probe (minimum length of 1 kb) to the corresponding chromosomal region of metaphase chromosomes or interphase nuclei (Tkachuk *et al.*, 1991). Both the labelled probe DNA and the chromosomal DNA

are denatured and incubated together at a temperature below the melting point of the probe/chromosomal DNA duplex. The probe is labelled prior to use (for example, by incorporating a biotin label by nick translation) and the location of the bound probe is revealed by fluorescent staining (for instance, by incubation with fluorescein-avidin that binds specifically to biotin), using fluorescence microscopy.

4.6 Use of Computational Sequence Analysis

Computational analysis has become a much valued research tool in molecular biology; this is reflected by the number of software programs available to analyse structural and functional characteristics of a given DNA sequence. Sequences can be analysed for homology to known sequences (eg BLAST and FASTA programs), possible intron-exon junctions and transcription-factor binding sites (eg PromotorScan, NetGene), or homology to known functional or structural motifs (eg BLOCKS, ProfileScan, MotifFinder). Common protein sequence motifs include nucleotide binding sites (ATP, GTP), phospho-transferase sites, zinc fingers, nuclear localisation sites, and modification sites (eg *N*-glycosylation, phosphorylation by protein kinases)(Harlow & Lane, 1988).

Homology searching is amongst the most widely used means of identifying new genes. The most informative option consists of sequence translation (into all six possible reading frames) and using the result as a query against databases containing amino acid sequences (such as the BLASTX program) or functional motifs. If homologs with known functions can be found, the researcher gains information at very little cost and time compared with experimental analysis. However, not all newly discovered proteins have homologs in databases; it appears that the proportion is approximately half (Fickett, 1996).

Information gained from computer homology analysis regarding potential functional motifs must be interpreted carefully; what level of homology to a given sequence is necessary before one can claim significant similarity? The criteria for finding potential functional motifs must carry sufficient stringency in order to avoid finding motifs which have no functional importance (Mikkelsen, 1993).

Within the current study, computational analysis was restricted by the length of sequence obtained and lack of available homologs; while EST walking allowed for the construction

of a 1.6 kb contig map, effectively only the 260 bp sequence isolated in this study could be used due to the amount of errors induced by the single sequencing reads typical of sequences submitted to the EST database. Homology searching is particularly sensitive to changes in frameshift introduced by the omission of actual bases or addition of bases not present in the sequence. Such sequencing errors are extremely common and can only be circumvented by purchasing the ESTs and performing additional sequence reads. This option was investigated but not followed through due to the cost of the additional sequencing and time involved, but would be extremely worthwhile if this gene was to be sequenced in its entirety.

Availability of the complete sequence would also allow for the use of intergrated gene-finding programs (eg GRAIL, FGENEH, GeneParser). These give an overall predicted gene structure based on a set of intergrated algorithms, or rules, about gene structure/function relationships. Intergrated gene-finding programs have greater prediction accuracy than programs which recognise isolated features of genes. These programs are most efficient when the input sequence encompasses exactly one entire gene and sequencing errors are absent. Furthermore, current algorithms cannot detect certain features of genes (eg alternative splicing and overlapping genes), and in some cases may not be particularly accurate (Fickett, 1996).

Computational methods can be extremely efficient, however, they rely on knowledge gained from experimental methods. As experimental methods become more powerful, it follows that the accuracy of computational analysis will increase also. In this sense, they are complementary and both will continue to play crucial roles in elucidating the structure and function of genes.

4.7 Open Reading Frame +1 Does Not Contain Stop Codons

Translation of the sequence obtained from the MCF-7 cDNA library into the six possible reading frames gave stop codons in all but one of the translations. This reading frame (ORF +1) contained several likely epitopes, ie regions with homology to hER aa 497-507 that could be recognised by antibody 15F6. These regions were selected based on amino acid identity and charge similarity.

In order to confirm that ORF +1 is the correct reading frame, it was envisaged that the 260 bp insert could be cloned into the expression vector pFLAG, the recombinant protein purified and subjected to N-terminal sequencing. In this case, a recombinant protein containing the correct reading frame can be detected on a Western blot by antibody 15F6 in addition to the pFLAG antibody. The latter recognises a small marker peptide (1 kD) which becomes fused to the N-terminus of the recombinant protein. The marker peptide is encoded by the pFLAG vector, hereby providing a means of detecting the recombinant protein (International Biotechnology, Inc., Catalogue Number IB 13000).

This strategy was attempted but proved unsuccessful (data not included). While the ligation was successful, the protein extracts made did not contain the appropriate size recombinant protein, as determined by SDS-PAGE and Western blotting.

4.8 Peptide 2 Contains the Epitope Recognised by Antibody 15F6

The antibody has the greatest affinity for peptide 2. This peptide is relatively long (25 residues) and contains 11 hydrophobic residues, resulting in poor solubility. The addition of acetic acid improved antibody binding but still left fine needle-like crystals in solution, indicating poor solubility. To further define the epitope and improve peptide solubility, a series of shorter, overlapping peptides could be made and tested by ELISA.

4.9 Potential Insight into Biological Function - Looking for Clues

The following experimental approaches could be of some use in elucidating aspects of the function of this novel antigen;

1. Examination of possible cell-cycle effects on the expression pattern of the antigen. This could be investigated by immunological staining of cells fixed during the different stages of the cell cycle. The observation that not all cell nuclei stain with similar intensities may provide some support for the notion that expression is cell-cycle linked.
2. Characterisation of the effect of growth factors, hormones and other regulatory factors on the expression pattern of the antigen, within a cell type of interest. This could involve exposure to the factor during cell culture and comparison to cells grown under normal culture conditions.

In a situation such as that presented by this research, many options become available for experimentation, the challenge lying with the researcher. Monoclonal antibodies lend themselves to a wide variety of methodologies, hereby permitting a large degree of experimental freedom.

At this time no further comment can be made about the biological significance of the antigen recognised by antibody 15F6. Gaining an understanding of its function is likely to involve a need to be able to detect and measure antigen levels, either for research or clinical purposes. Should this become the case, monoclonal antibody 15F6 will be an extremely valuable tool.

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Appendix A : Reagents and Materials

Source of Reagents

All reagents used were of analytical (AR) grade and obtained from the following suppliers;

BDH Chemicals Ltd., Poole, England
Sigma Chemical Co., St Louis, Missouri, USA
Amersham, Buckinghamshire, England
Gibco Life Technologies, Grand Island, New York, USA
Boehringer Mannheim, D-6800 Mannheim 31, West Germany
Pharmacia, Fine Chemicals AB, Uppsala, Sweden
Bio-Rad Laboratories, Hercules, California, USA

Source of Materials

Amersham, Buckinghamshire, England
Bio-Rad Laboratories, Hercules, California, USA
Boehringer Mannheim, D-6800 Mannheim 31, West Germany
Chiron Mimitopes Pty. Ltd., Clayton, Victoria 3168, Australia
Dako, Carpinteria, CA 93013, USA
Eastman Kodak Co., New York, USA
Epicentre Technologies, Madison, WI 53713, USA
Gibco Life Technologies, Grand Island, New York, USA
IDS, Usworth Hall, Washington, NE37 3HS, England
Irvine Scientific, Santa Ana, California, USA
LI-COR, Inc., Lincoln, Nebraska 68504, USA
Millipore Corporation, Bedford, MA
Multiple Peptide Systems, San Diago, California, USA
Nunc, Inc., 2000 North Aurora Road, Naperville, IL 60566

Pharmacia, Fine Chemicals AB, Uppsala, Sweden

Schleicher & Schuell, West Germany

Sigma Chemical Co., St Louis, Missouri, USA

Tago, Inc., Burlingame, CA, USA

Appendix B : Buffers and Media

A.2.6 Enzyme-linked Immunosorbent Assay (ELISA)

Phosphate Buffered Saline (PBS) Assay Buffer
10 PBS tablets
1 ml Tween 20
1 g gelatin (dissolved in 100 ml distilled water by heating in microwave)
Made up to 1 l with distilled water

Wash Buffer
23.4 g $NaH_2P0_4.2H_20$
26.4 g NaCl
3 ml Tween 20
Made up to 3 l with distilled water, pH adjusted to 7.4 with 5 M NaOH

Substrate Buffer
7.1 g Na_2HPO_4
5.25 g citric acid
Made up to 1 l with distilled water

Substrate
50 ml substrate buffer
20 mg OPD (*o*-phenylenediamine dihydrochloride)
30 µl hydrogen peroxide 30% w/v
Prepared before use and stored in dark

A.2.8 Immunological Staining

Phosphate Buffered Saline (PBS)
1 PBS tablet for every 100 ml of distilled water

0.1 M Tris-HCl Buffer
12.1 g Tris
300 µl H_2O_2 (30% w/v)
Made up to 1 l, pH adjusted to 7.6 with conc HCl

Diaminobenzidine (DAB) Substrate
50 mg DAB
100 ml 0.1 M Tris-HCl buffer

Fast Blue substrate
2 mg sodium AS phosphate dissolved in 100 µl dimethyl formamide (DMF)
10 mg fast blue salt
6 µl 1 M levamisole

10 ml 0.1 M Tris, pH 8.2

AEC substrate
10 mg AEC dissolved in 100 µl of DMF
10 ml 0.05 M acetate, pH 5.1

A.2.9 Binding Studies

Tris-EDTA Buffer
1.21 g Tris
0.558 g EDTA
4.84 g Na_2Mo0_4
154 mg dithiothreitol
100 ml glycerol
Made up to 1 l with distilled water, pH adjusted to 7.4 with conc HCl.

A.2.10 Western Blot Analysis

Sample buffer
4.0 ml distilled water
1.0 ml 0.5 M Tris-HCl, pH 6.8
80 µl glycerol
1.6 ml 10% (w/v) SDS
0.4 ml 2-b-mercaptoethanol
0.5 ml 0.05% (w/v) bromophenol blue
Added in equal volumes to sample and heated at 95°C for 4 min before loading

Electrophoresis buffer (5X)
15 g Tris
72 g glycine
5 g SDS
Made up to 1 l with distilled water, pH adjusted to 8.3 and diluted to 1x before use

Transfer buffer
6 g Tris
28.8 g glycine
1.8 L distilled water
200 ml methanol

Tris-buffered saline (TBS)
6.5 g Tris
9 g NaCl
1 ml Tween 20
Made up to 1 l with distilled water, pH adjusted to 7.4 with conc HCl

Enhanced chemiluminesce substrate (ECL)
This substrate was prepared by adding equal volumes of solutions 1 and 2:

Solution 1
100 ml 0.1 M Tris, pH 8.6
400 µl luminol stock (44 mg 5-amino-2,3 dihydro-1,4-phthalazinedione in 400 µl dimethylsulfoxide (DMS0))
24 µl acid stock (0.09 g 4-hydroxycinnamic acid in 1 ml DMSO)

Solution 2
100 ml 0.1 M Tris, pH 8.6
80 µl 30% w/v H_2O_2

A.2.12 Affinity Chromatography

TED buffer
2.42 g Tris
372 mg EDTA
154 mg dithiothreitol 1mM
Made up to 1 l with distilled water, pH adjusted to 7.4 with conc HCl.

A.2.14 Immunodiffusion and Immunoelectrophoresis

Tris-barbitone buffer
3 g barbituric acid
4.6 g Tris
8.9 g Na barbitone
0.4 g Ca lactate
Made up to 1 l with distilled water, pH adjusted to 8.8 with conc HCl.

Coomassie Blue
10 g Coomassie Brilliant Blue
450 ml 70% ethanol
100 ml glacial acetic acid
450 ml distilled water

Destain
95 ml distilled water
95 ml 70% ethanol
10 ml glacial acetic acid

A.2.15 Media

Luria Broth (LB)
20 g Bacto Tryptone

10 g yeast extract
20 g NaCl
Made up to 2 l with distilled water, pH adjusted to 7.0 with 5 M NaOH, and autoclaved.

Ampicillin stock solution
15 mg/ml amp stocks were made up in distilled water. Stocks were filter-sterilised and stored at -20°C for a maximum of 3 weeks.

LB + Amp plates
300 ml LB
4.5 g agar
This was heated in a microwave until all agar had dissolved (approx. 6 min) and allowed to cool. When cooled to 50°C, 1 ml amp stock was added and plates made by pouring 25 ml of media per petrie dish. Plates were left to set, inverted, and stored at 4°C for up to 2-3 weeks. Prior to use, plates were dried inverted, without lids, for 1 h at 37°C.

A.2.16 Antibody Screening of the MCF-7 cDNA Library

SM buffer
5.8 g NaCl
2 g $MgSO_4.7H_2O$ or 0.98 g anhydrous $MgSO_4$
6.5 g Tris
0.2 g gelatin
Made up to 1 l with distilled water, pH adjusted to 7.5 with conc HCl, and autoclaved.

Alkaline phosphatase (AP) buffer
12.1 g Tris
5.9 g NaCl
1.2 g $MgCl_2.6H_2O$
Made up to 1 l, pH adjusted to 9.5 with conc HCl

Alkaline phosphatase substrate
Stock solutions
1. 50 mg/ml BCIP in 100% dimethylformamide (DMF)
2. 50 mg/ml NBT in 70% dimethylformamide

100 ml AP buffer
660 µl NBT stock
330 µl BCIP stock
Substrate was freshly prepared before use.

Peroxidase substrate
80 mg 4-chloro-1-napthol dissolved in 20 ml methanol
120 µl H_2O_2
80 ml TBS
Substrate was freshly prepared before use.

A.2.19 Restriction Digestion of λgt11 Bacteriophage DNA

TBE electrophoresis buffer (10 X stock)
108 g Tris
55 g boric acid
3.7 g EDTA
Made up to 1 l with distilled water.

Agarose gels
1% agarose gels were made by adding 500 mg agarose to 50 ml TBE (1 X) buffer in a conical flask. The agarose was heated until completely dissolved (2 min in a microwave), and the gel cooled to approximately 50°C before pouring. Gels were allowed to set for 45 min to 1 h before use.

Loading dye (6 X)
25 mg bromophenol blue
25 mg xylene cyanol FF
6 ml distilled water
4 ml sucrose

Ethidium bromide
Made up as a 10 mg/ml stock solution. 5µl of stock solution was added to the TBE electrophoresis buffer prior to use.

TE (100 X stock)
12.1 g Tris
3.7 g EDTA
Made up to 100 ml with distilled water, pH adjusted to 8.0 with conc HCl, and autoclaved.

Restriction digests
DNA digests were performed in microcentrifuge tubes in either a 10µl or 20µl volume, containing 10x buffer (one-tenth of final volume), RNAse (1 µl of 10mg/ml stock), restriction enzyme (1-2µl at 10U/µl), DNA, and distilled water (to 10µl or 20µl). Digests were performed at 37°C for 65m min in a heated water bath, after which the reaction was stopped at 75°C for 10-15 min in a heated block.

A.2.21 Plaque Lift and Library Screening

Denaturation solution
43.8 g NaCl
10 g NaOH
Made up to 500 ml with distilled water

Neutralisation solution
43.8 g NaCl
60.55 g Tris
Made up to 500 ml with distilled water, pH adjusted to 8.0 with conc HCl.

Modified Church buffer
Equal volumes of;
10 % SDS
0.5 M Na_2HPO_4
Preheated to 65°C.

20 x SSC (Standard Saline Citrate)
175.32 g NaCl
88.23 g sodium citrate
Made up to 1 l with distilled water, pH adjusted to 7.0 with NaOH.
Diluted as necessary with distilled water (ie 2 x SSC, 0.1 SSC).

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Postscript

On the 10th of March 1997, a final BLAST sequence search was made. 100% homology was reported between the sequence obtained in this study and part of the human mRNA for ataxin 2, also known as spinocerebellar ataxia type 2 protein (SCA2). Agreement was also found between the deduced reading frame, molecular weight, location and expression pattern given here and published information. The function of this protein remains unknown, however, its abnormal expression has been implicated in the neurodegenerative disorder spinocerebellar ataxia type 2, one of the dominant spino-cerebellar ataxias (SCAs)[1-3].

SCAs are part of a class of dominantly inherited neurodegenerative disorders, including Huntington's disease (HD) (ref in Sanpei), spinobulbar muscular atrophy (SBMA), and dentatorubral-pallidoluysian atrophy (DRPLA). The mutation responsible for these disease phenotypes is expansion of an unstable trinucleotide repeat (CAG), encoding a polyglutamine stretch in the corresponding protein. The affected proteins are dissimilar except for the polyglutamine stretches, and are thought to have different functions.

One of the strategies used to clone the *SCA2* gene was expression-linked cloning, using a monoclonal antibody raised against the polyglutamine containing transcription factor TBP (TATA binding protein)[1]. This antibody has been shown to detect the pathological proteins present in HD, SCA1 and SCA3 patients using western blotting[4]. Western blots give an apparent size of 150 kD for the mutant SCA2 protein, however, it has been suggested that a size of about 120-130 kD is more realistic, given that expanded polyglutamine stretches are known to affect electrophoretic migration[1]. This is in agreement with the molecular mass predicted by examining cDNA structure; a mass of 124 kD or 140 kD has been suggested depending on which one of two start (ATG) codons is utilized. The ATG codon predicted to give rise to the 124 kD product is in better agreement with Kozak's consensus sequence[2].

Western blot analysis of fractionated lymphoblastoid cell lines reveals the presence of the SCA2 protein in the cytosolic fraction[4]. A similar result using western blotting was obtained in the current study, however, immunological staining shows a predominantly

nuclear localisation. It is likely that the protein is loosely associated with the nucleus under normal cellular conditions, partitioning into the cytosolic fraction under fractionating conditions for detection with western blotting.

The expression pattern reported here is in agreement with Northern blot analysis. A 4.5 kb transcript was detected in brain, heart, placenta, liver, skeletal muscle and pancreas. The transcript was absent from lung and kidney[2,3]. No further tissues were tested.

In conclusion, the evidence suggests that monoclonal antibody 15F6 specifically recognises the SCA2 protein. This makes it an invaluable research tool with applications which could provide insight into the biology of SCA2.

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